Production of L-methionine with *Corynebacterium glutamicum*

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1. Referent: Prof. Dr. Siegmund Lang
2. Referent: Prof. Dr. Klaus Dieter Vorlop
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Abstract

Targets of this project were the screening for microbial overproducers of L-methionine by random mutation (UV-radiation plus following selection) and optimization of the production in shake flask and bioreactor scale by varying of biochemical (nutrient concentration, pH) and general cultivation parameters (temperature, stirring speed, in bioreactor: oxygen partial pressure and fed batch).

In this context one major focus of the research was the development of a fast screening and selection system for microbial overproducers of L-methionine. For the necessary experiments the strain *Corynebacterium glutamicum* DSM20300 was used. A big problem was the selection system after radiation with ultraviolet light (UV) to sort out potential candidates for the analysis. Although a successful inhibition of non-methionine producing microorganisms using methionine analogues like ethionine had been described in former experiments (Mondal et al. 1996), it was not possible to reproduce these results. Due to this problem in the selection method the measurement of a huge amount of colonies obtained after the UV-radiation could not be avoided. In spite of all efforts undertaken to accelerate the analysis methods, the chances to find overproducers in screening experiments were noticeable diminished. The attempt to find a microbial overproducer in these screening experiments has not been successful.

A second challenge was the searching for microorganisms already containing the property to overproduce L-methionine. One strain, *Corynebacterium glutamicum* ATCC21608, is commercially available from a public collection of microorganisms in the USA. It could be shown in several experiments, that this bacterium is able to produce L-alanine in small amounts (200 mg/l) but L-methionine could not be produced in significant quantities.

The strain *Corynebacterium glutamicum* KY10574 has been obtained from the company Kyowa Hakko Kogyo, Japan, a well-known producer of amino acids with microorganisms. A successful accumulation of L-methionine was observed. In first experiments the concentration of L-methionine in the supernatant of the cultivations was about 100 – 150 mg/l, in further experiments in the shake flask (several scales) and the bioreactor (3 – 3.5 litres) L-methionine concentrations of 1.4 – 1.5 g/l could be achieved. For both bioreactor and shake flask scale significant parameters for this optimization were the creation of an adequate minimal medium and the oxygen supply in combination with the stirring speed. In bioreactor scale the best process was the cultivation with a limitation factor (sugar, glucose) in order to be able to control cell growth velocity and guarantee aerobic conditions through efficient oxygen supply. L-methionine was only produced in higher amounts under aerobic conditions in the cultivation broth.

Abstract

Ziel dieses Projektes war das Screening nach mikrobiellen Überproduzenten der Aminosäure L-Methionin durch zufallsbasierte Mutagenese (UV-Bestrahlung und nachfolgende Selektion). Daraufhin sollte die Produktion im Schüttelkolben- und Bioreaktormaßstab durch Variation der biochemischen (Nährstoffkonzentrationen, pH) und generellen Kultivierungsparameter (Temperatur, Schüttel- und Drehzahlraten, im Bioreaktor: Sauerstoffpartialdruck und Zufütterungsstrategien) optimiert werden.

Zunächst war die Entwicklung eines schnellen und effizienten Screenings- und Selektionssystems für mikrobielle Überproduzenten von L-Methionin das Hauptziel des Projektes. Dafür wurde der Stamm *Corynebacterium glutamicum* DSM20300 benutzt. Als grosses Problem stellte sich die Etablierung eines effizienten Selektionssystem zum Aussortieren potentieller Kandidaten für die Aminosäureanalyse heraus. Obwohl eine erfolgreiche Inhibierung mikrobiellen Wachstums mit Methioninanaloga wie Ethionin in der Literatur beschrieben worden ist (Mondal et al. 1996), war es nicht möglich diese Ergebnisse zu reproduzieren. Aufgrund dieser Probleme mit der Selektionsmethode konnte das Vermessen einer grossen Menge von Mutantenkolonien, die nach UV-Bestrahlung erhalten worden sind, nicht vermieden werden.

Trotz aller Versuche die Analytik zu beschleunigen, waren die Chancen einen Überproduzenten in den Screeningexperimenten zu finden, dadurch sehr verringert. Es war im weiteren Verlauf der Experimente nicht möglich einen L-Methionin Überproduzenten herzustellen.

Ein weiterer Punkt war die Suche nach Mikroorganismen, die bereits die Fähigkeit zur Produktion von Methionin besitzen. Der Stamm *Corynebacterium glutamicum* ATCC21608 ist kommerziell an einer amerikanischen Stammsammlung erhältlich. In verschiedenen Versuchen konnte gezeigt werden, dass dieser Stamm die Fähigkeit zur L-Alaninproduktion in Mengen um die 200 mg/l besitzt. L-Methionin wurde von diesem Stamm nicht überproduziert.

Der Stamm *Corynebacterium glutamicum* KY10574 wurde von der Firma Kyowa Hakko Kogyo, Japan, Tokio zur Verfügung gestellt. Hier konnte eine L-Methioninproduktion beobachtet werden. In den ersten Untersuchungen konnten L-Methionin Konzentrationen zwischen 100 – 150 mg/l im Überstand gemessen werden, in weiteren Experimenten im Schüttelkolben- und Bioreaktormaßstab wurden Konzentrationen zwischen 1,4 und 1,5 g/l erzielt. Wichtige Parameter für diese Optimierung waren die Herstellung eines adäquaten Minimalmediums, die Sauerstoffversorgung und die Zufütterungsstrategie (Glucose) im Bioreaktor. L-Methionin wurde nur bei ausreichendem Sauerstofftransfer in das Kultivierungsmedium in höheren Mengen produziert.

1 Introduction

Apart from l-lysine and l-threonine, L-methionine belongs to the essentiell amino acids which human and animal metabolisms are not capable to produce. Most natural feeds as wheat or maize protein, soya bean and fish meal are deficient in methionine, lysine and threonine. Therefore these amino acids are the most important nutrient additives in animal feeding. For pig and poultry breeding specific feeding plans exist with L-methionine as essentiell and sulphur containing amino acid. The impact of L-methionine on animal nutrition and the consequences of its absence as nutritive feed additive have been investigated very well. It has been observed for poultry that the stability of egg shells decreases just as the milk production in cows does (Noftsger et al. 2003; Keshavarz et al. 2003).

In 1995 the demand for methionine amounted to 300,000 tons per year (Leuchtenberger et al. 1996). The general and cheapest process to obtain L-methionine is the chemical synthesis using acroleine, methyl mercaptan and hydrocyanic acid (Leuchtenberger 1996; Pack 2004). The product is a racemic mixture of D- and L-methionine. Another method to obtain L-methionine is the extraction from protein hydrolysates (Kircher et al. 1998). In 1998 500,000 tons D/L-methionine per year were produced (Toride 2002), the market for amino acids in general and for L-methionine in special increases constantly.

Normally only the L-form can be utilized by human and animal metabolisms, but for methionine enzymes are available in human and animal bodies which make it possible to convert the D-form of the chemically synthesized racemic DL-methionine mixture. The organism converts the D-form enzymatically into the nutritive L-form via an amino acid oxidase system by oxidative desamination and subsequent transamination reactions (Leuchtenberger et al. 2005; Hasegawa et al. 2005). This is an enormous potential for the manufacturer to reduce costs and the reason why there is no other process to produce methionine and replace the chemical production up to the present.

However, a new EU ordinance from August 2005 prohibits the use of synthesized methionine for animal feed addition in ecological farming. Due to this law new alternative methods to obtain methionine from ecological resources will need to be developed. All current work about *de novo* synthesis of L-methionine in the European Union with renewable primary products like sugar and starch are based on genetic engineering (directed mutations) of the

metabolic pathway and the modification of the enzyme activities and the intermediates / precursors which occur in the metabolic pathway of L-methionine (e.g. Mampel et al. 2005).

A model organism for the overproduction of amino acids in microorganisms is *Corynebacterium glutamicum*, because of its simplified metabolic pathways for the production of amino acids (Lee et al. 2003; Gomes et al. 2005). For this reason it is easier to develop a new strain with changed metabolic fluxes in order to produce L-methionine. The production of L-lysine, another important animal food additive, is performed also with overproducing strains of *Corynebacterium glutamicum* (Eggeling et al. 1999). For L-lysine, it is the state of the art technique.

Most publications about L-methionine production mention about the screening system which leads to microbial overproducer mutants. The problem is, that the production of amino acids in microorganisms is subject to a feedback inhibition, which prevents overproduction (Wartenberg 1989). For this reason the first target in research is to find a way to deactivate this molecular mechanism. The first and traditional method is the random mutation with following selection (Gerhardt et al. 1981). The treatment of cells with UV-radiation or chemical mutation agents (random mutation) and the selection with methionine analogues / antimetabolites is widely described in the literature (Kase et al. 1974; Mondal et al. 1996; Kumar et al. 2003).

Over the last 20 years, genetic engineering with directed mutation methods has developed into another approach with increasing potential. The knowledge of pathways and mechanisms from gene to product allows to work specifically through the activating or inactivating of genes and enzyme activities. Due to gene technology it is possible to change the metabolic flux in microorganisms step by step and canalise it in the designated direction to reach significant amounts of a special metabolite.

Today it is common to make attempts to get microbial overproducers with both random and directed mutation methods. The experiences in the production of other amino acids like L-lysine and L-glutamic acid (e.g. Eggeling et al. 1999) will be of great value for the research on L-methionine production in the European Union (EU) in the next years in order to develop microbial overproducers and economical biotechnical processes for L-methionine production.

2 Theoretical background

2.1 Amino Acid Production

The production of amino acids is a big industrial factor in both the chemical and biotechnological industries. There has been always hard competition between these two fields to produce amino acids in a cheap and energy reducing mode.

Amino acids have many special properties which make them very valuable, as for example their contribution to nutrition, the taste, the chemical features and their importance in physiological activities. The proteinogenic amino acids are the building blocks of proteins, they are important intermediates on the pathway from the genetic to the protein level.

The varied use of amino acids are as supplements to human and animal food, medical infusions, cosmetics and intermediates in the chemical industry. According to data from 1995 the whole market is estimated at 3 billion US $ in 1995. divided in 38% for food, 54% for feed and 8% for other applications (Leuchtenberger et al. 1996). Figure 2.1 shows an overview of all methods to gain amino acids in industry.

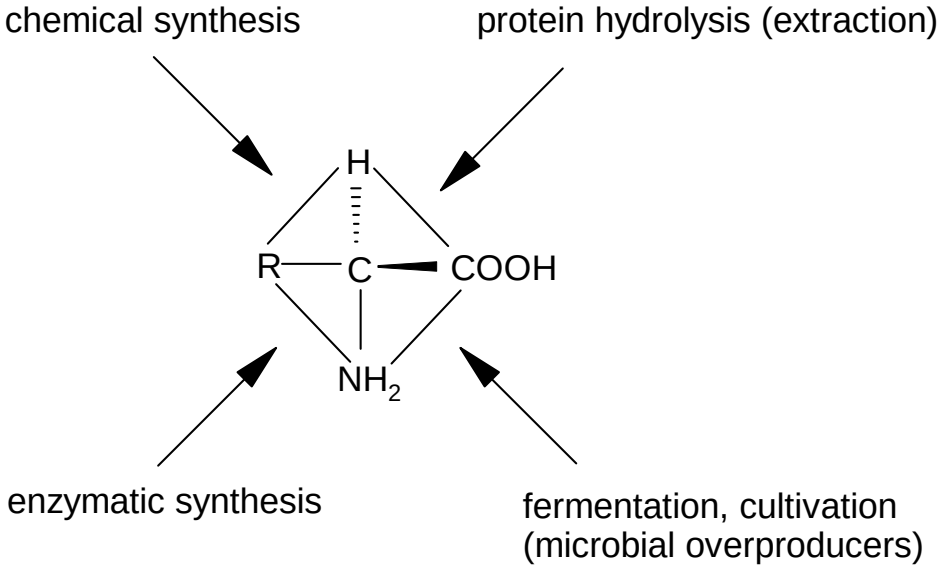


Figure 2.1: Principle possibilities to produce L-amino acids or D/L-amino acids in the case of chemical synthesis (modified according to Leuchtenberger et al. 1988).

It is possible to synthesize all amino acids in the traditional chemical way but for many of them it would be much more profitable to produce with different methods. The advantage of the enzymatic synthesis and the direct fermentation is the modern enantioselective production of either the L- or D-enantiomeric form. There are examples for each of the production possibilities mentioned in Figure 2.1. Glycine is the only nonchiral amino acid, therefore the chemical process is without competition because there is no racemic product mixture to purify. For L-methionine, the chemical synthesis in combination with the enzymatic resolution of the racemic mixture is the most important form of production.

L-asparagine, L-arginine, L-histidine and L-cysteine for example are produced by extraction from protein hydrolysates; L-tryptophan and L-aspartic acid are obtained using enzymes or immobilized cells.

The barrier for multi enzyme systems is reached when the effectiveness of the microbial cell as enzyme membrane reactor is much higher in spite of side reactions and by-products. On this account the direct fermentation is the preferable process in commercial aspects for L-lysine and L-glutamic acid (Kole et al. 1986; Kinoshita et al. 1961; Kiefer et al. 2004).

A major problem is the strong regulated biosynthesis in wild type microorganisms. The produced amino acid itself restricts the formation of necessary enzymes (feedback repression) and / or reduces the activity of key enzymes for the metabolic building pathway (feedback inhibition) (Leuchtenberger et al. 1988). In a suitable strain the control mechanisms have to be deactivated. In addition, side reactions and the degradation of end and intermediate products have to be blocked. The export and discharge of the product to the extracellular environment needs to be considered, too (Krämer 1993; Trötschel et al. 2005).

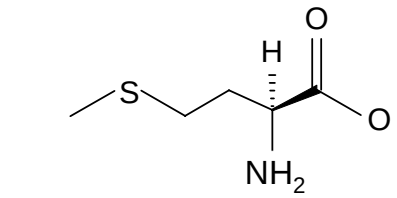
2.2 L-methionine

Methionine (CAS registry number 63-68-3) occurs in two enantiomeric chiral forms. L-methionine is the proteinogenic one. It has nonpolar properties and is, apart from cysteine, the unique amino acid with a sulphur component. In normal environment conditions it is a solid white powder. Other physicochemical properties and the molecular structure are shown in the Table 2.1 and Figure 2.2. According to the metabolic pathway L-methionine belongs together with L-lysine and L-threonine to the aspartic group of amino acids. These 3 proteinogenic

acids, which are all of great importance for human and animal food, become synthesized from aspartic acid, the first corporate reaction is catalyzed by aspartate kinase, an enzyme only occurring in plants and microorganisms (Voet et al. 2002).

Table 2.1: Physicochemical properties of the amino acid L-methionine (Barrett et al. 1985)

IUPAC – IUB abbreviations	Met; M
Molecular weight	149.22
Decomposition temperature	283
Water solubility (g/100g; 25°C)	3.5
pK_{COOH}	2.28
pK_{NH3}^{+}	9.21
Isoelectric pH	5.74
Density (g/cm^3)	1.34
Taste in aqueous solution	tasty



$C_5H_{11}NO_2S$

Figure 2.2: Molecular structure and empirical formula of the amino acid L-methionine.

In metabolism L-methionine is a provider of methyl groups, e.g. for choline, adrenaline and nucleic acid biosynthesis. It is an essentiell amino acid for humans and animals, so it is important to get enough methionine through nutrition. The bioactive form is S-adenosyl-methionine (SAM) (Kase et al. 1975; Schlenk et al. 1978), an important donor of methyl groups in organisms.

In the biologic translation system the start of the protein biosynthesis always begins with the 3 nucleotides for L-methionine (AUG) as starting codon; but later a detachment of L-methionine from the constructed poly peptide chain is often observed.

2.2.1 DL-methionine production by chemical synthesis

The most important industrial method to produce DL-methionine is the chemical synthesis. It is also the most economical until now. This is due to the fact that it is possible to use the racemic mixture for feeding additives because the human and animal organisms are able to convert the D-form of methionine completely into the nutritive L-form by means of an oxidase and transaminase (Hasegawa et al. 2005).

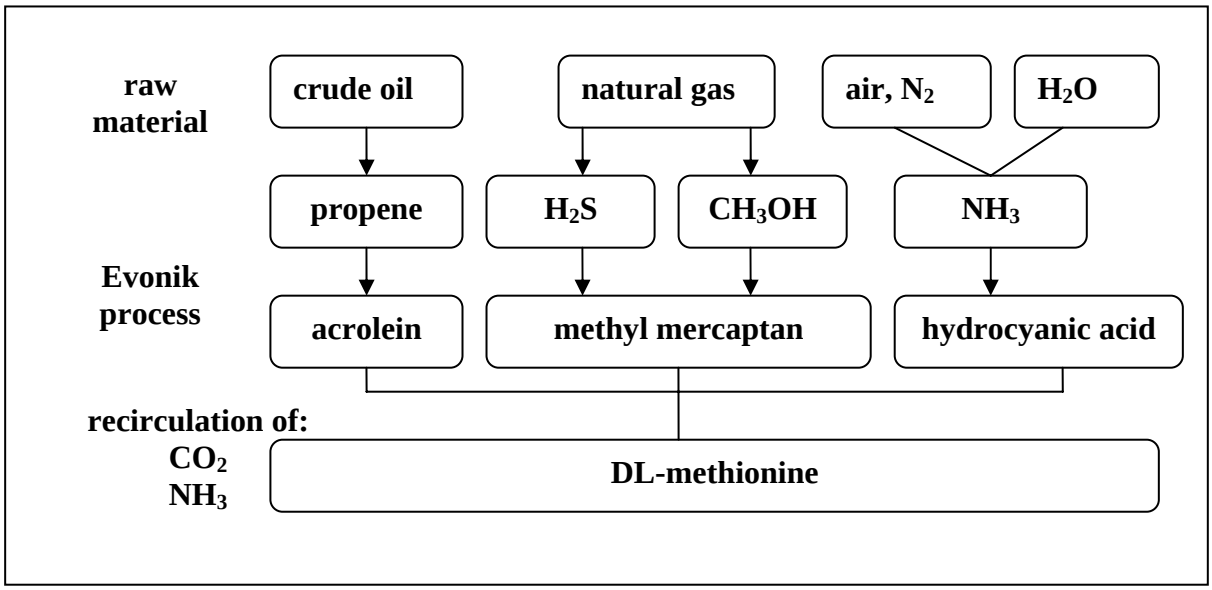


Figure 2.3: Diagram of the chemical synthesis of DL-methionine by Evonik (former Degussa). Apart from L-methionine Evonik produces also the substrates acrolein, methyl mercaptane and cyanic acid (Pack 2004).

Figure 2.3 shows a description of the DL-methionine synthesis of the Evonik (former Degussa) company in Antwerpen. The most fundamental raw materials are crude oil and natural gas, in addition basic chemicals like propene, methanol, ammonia and hydrogen sulphide are used. The process has to pass several intermediate steps. At the end the product is obtained through a reaction of acrolein, methyl mercaptan and hydrocyanic acid. The advantage of this procedure is the relative high yield and the high rate of recirculation of carbon dioxide and ammonia. The ressource consumption is comparatively low: per kilogram amino acid only one kilogram crude oil has been consumed (Degussa 2004).

2.2.2 L-methionine production by enzymatic synthesis

For pharmaceutical purposes an important industrial process to gain L-methionine is the acylation of DL-methionine with the enzyme L-aminoacylase, which catalyses the hydrolysis reaction from DL-acetylated amino acids to L-amino acids. The reaction is enantioselective; so it is easy to separate the L-forms from the D-forms which are furthermore in the acetylated condition. This process was first performed by the Japanese company Tanabe in 1969 to get L-amino acids on an industrial scale (Leuchtenberger et al. 1988). Also, in recent research there are studies about optimizing the conversion of the racemic mixture into pure L-amino acids (May et al. 2000; Tokuyama et al. 1996).

Another enzymatic catalysis which was performed in the 1990s is the conversion of DL-methyl-thio-ethyl-hydantoin with *Arthrobacter aurescens* DSM7330 to L-methionine (Voelkel 1993; Stehr 1996), described in Figure 2.4.

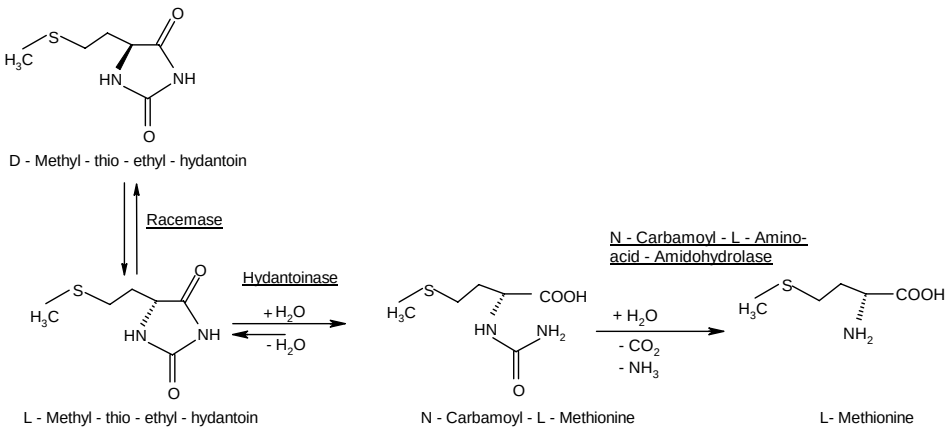


Figure 2.4: Description of the molecular mechanism for the conversion from DL-methyl-thio-ethyl-hydantoin (MTEH) to L-methionine with *Arthrobacter aurescens* DSM7330 (modified according to Stehr 1996).

The hydantoin hydrolysis is an economic synthesis for optical pure amino acids. Several hydantoin derivatives as substrates can be received in cost-efficient ways by chemical synthesis. A complete conversion by enzymatic catalysis is assured, there are no costs for derivatization and racemization (Voelkel 1993).

Three enzymes are responsible for the formation of the L-amino acids, in Figure 2.4 shown e.g. for the production of L-methionine (Stehr 1996):

- hydantoin-racemase: catalyzes the racemization from D- to L-hydantoins

- hydantoinase: catalyzes the hydrolysis of the amide bond of the hydantoin circle
- N-carbamoyl-amino-acid-amidohydrolase: catalyzes the release of the amino acid by separation of carbon dioxide and ammonia from carbamoyl intermediate product.

Important for this reaction is the activation of enzyme activity with the aid of an inducer, in this case DL-N-3-methyl-5-indolyl-methyl-hydantoin (IMH). Without treatment of the cells with this inducer the production rate of L-methionine would be ten times lower (Stehr 1996).

2.3 Corynebacterium glutamicum

2.3.1 General information

In 1957 Kinoshita et al. isolated a bacterial strain which was able to overproduce L-glutamic acid in minimal media with glucose as carbon source and release the product in the extracellular environment. The isolated soil bacterium was named *Corynebacterium glutamicum*. In taxonomic terms it belongs to the family of *Corynebacteriaceae*. Its cell wall formation is very characteristic (gram positive), especially the existence of mycolic acids which surround the entire cell as a structured layer (Eggeling et al. 2001). The wild type strains are mostly able to grow aerobically on basic minimal media containing a carbon source like glucose, phosphate, sulphate, ammonia and in addition biotin due to the fact that this bacterial species is completely biotin deficient (Stansen 2005). Furthermore *Corynebacterium glutamicum* is immobile and non-sporulating. Since the isolation in 1957 high amounts of L-glutamic acid have been produced with new developed or advanced strains of this species.

The cell wall of *Corynebacteriaceae* has a special structure which is different from other gram positive bacteria; this is shown in Figure 2.5. As you can see there, the peptidoglycan layer is connected to the heteropolysaccharide arabinogalactan. The external mycolic acid layer is linked again with the arabinogalactan (Eggeling et al. 2003). The influence of this cell wall formation on the export of amino acids is not yet well defined, the research on this field is described in chapter 2.3.5.

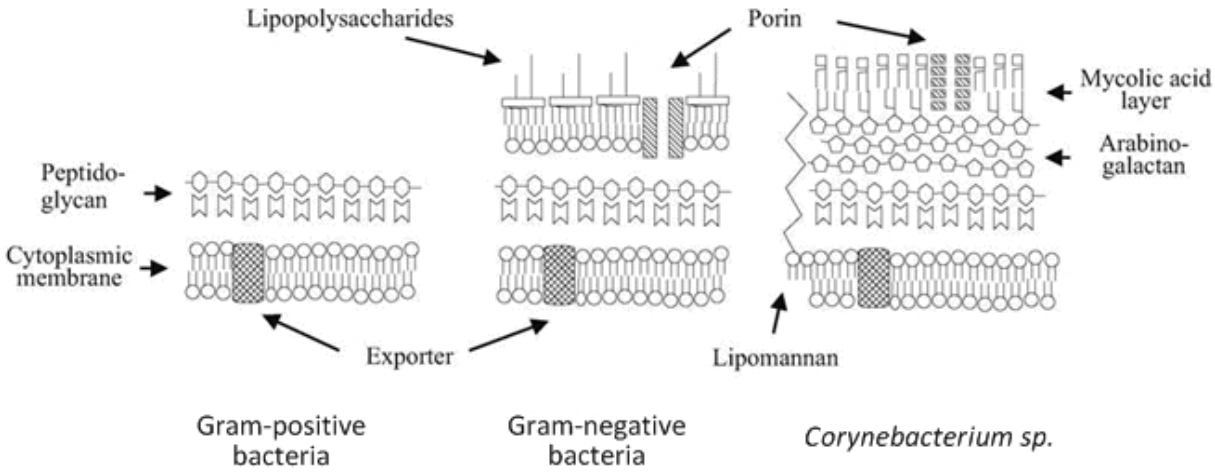


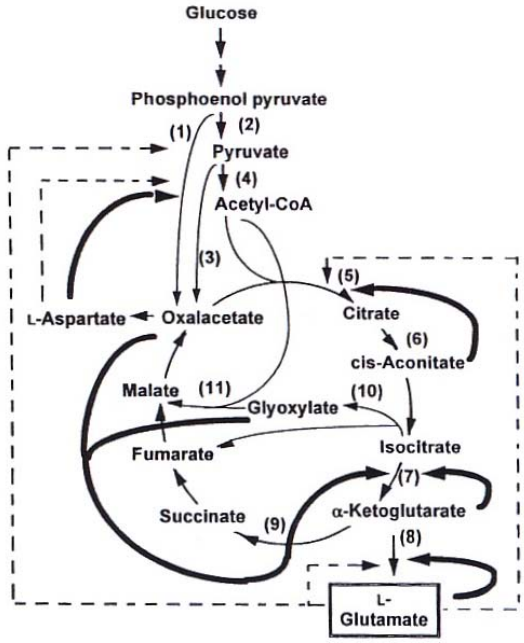
Figure 2.5: Formation of the *Corynebacterium glutamicum* cell wall (right) in comparison to gram-positive (left) and gram-negative bacteria (middle) (Eggeling et al. 2003).

Because of many experiences the scientists gained over the last decades about this organism and its metabolic fluxes in context of amino acid production, *Corynebacterium glutamicum* has become the most important bacterial strain for amino acid overproduction (Schmid 2002). It has been observed that the regulatory system is much more simple than that of *Escherichia coli* (Tosaka et al. 1986). There is information of the production of L-glutamic acid (e.g. Kinoshita et al. 1961), L-phenylalanine (e.g. Wartenberg 1989), L-lysine (e.g. Eggeling et al. 1999), L-valine (Blombach et al. 2007) and L-methionine (e.g. Kumar 2005) using strains of *Corynebacterium glutamicum*.

Due to the historical importance of this particular field of research, a short description of metabolic pathways for L-glutamic acid and L-lysine will be given in the following chapters 2.3.2 and 2.3.3.

2.3.2 L-glutamic acid production with *Corynebacterium glutamicum*

The process to gain L-glutamic acid with *Corynebacterium glutamicum* by direct fermentation (Kinoshita et al. 1961) is very well investigated. Until today there is research about some aspects of this topic (Stansen 2005). Key factors for the cultivation process in order to reach high amounts of L-glutamic acid are the optimal concentration of biotin to influence and support cell growth and the secretion of the product in the extracellular environment (Clement et al. 1986; Stansen 2005). Another important factor to prevent side reactions and by-products is the oxygen supply. Under partially anaerobic conditions other / additional products like lactic acid could be obtained (Kole et al. 1986).



Enzymes in Fig.2.6 (see numbers)

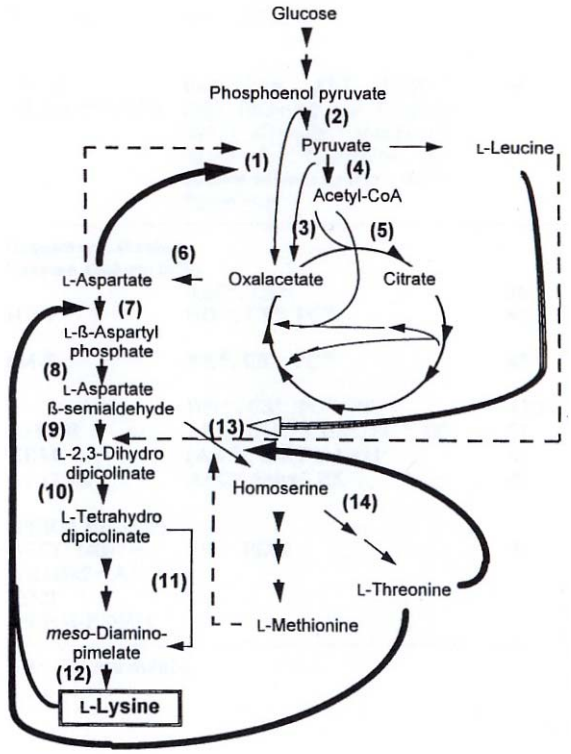
1: phosphoenolpyruvate carboxylase
2: pyruvate kinase
3: pyruvate carboxylase
4: pyruvate dehydrogenase
5: citrate synthase
6: aconitase
7: isocitrate dehydrogenase
8: L-glutamate dehydrogenase (GDH)
9: α-ketoglutarate dehydrogenase (KDH)
10: isocitrate lyase
11: malate synthethase

Figure 2.6: Regulation of L-glutamic acid biosynthesis in *Corynebacterium glutamicum* (Leuchtenberger 1996); straight lines represent feedback inhibition, dashed lines represent feedback repression.

The most important factor for L-glutamate overproduction is the activity of the enzymes GDH and KDH (see Figure 2.6). In overproducers the conversion velocity of α-ketoglutarate to L-glutamic acid with GDH is 150 times higher than the side reaction of the substrate with KDH which leads back to the citric acid cycle (Shiio et al. 1980). In Figure 2.6 the versatile regulation mechanisms in biological pathways for L-glutamic acid (feedback inhibition and repression) are shown, the problems in modifying these metabolic fluxes in desired directions are quite obvious due to the complexicity and various connections in these metabolic cycles.

2.3.3 L-lysine production with *Corynebacterium glutamicum*

In contrast to L-glutamic acid production the wild types of *Corynebacterium glutamicum* are not able to overproduce L-lysine, thus much research has been performed on modifying these organisms with directed and random mutations to obtain auxotrophic and regulatory deficient strains which are appropriate for L-lysine overproduction (Hilliger 1991).



Enzymes in Fig.2.7 (see numbers)

1: phosphoenolpyruvate carboxylase
2: pyruvate kinase
3: pyruvate carboxylase
4: pyruvate dehydrogenase
5: citrate synthase
6: L-aspartate aminotransferase
7: aspartate kinase
8: aspartate-4-semialdehyde dehydrogen.
9: dihydropicolinate synthase
10: dihydropicolinate reductase
11: m-diaminopimelate dehydrogenase
12: m-diaminopimelate decarboxylase
13: homoserine dehydrogenase
14: homoserine kinase

Figure 2.7: Regulation of L-lysine biosynthesis in *Corynebacterium glutamicum* (Leuchtenberger 1996); straight lines represent feedback inhibition, dashed lines feedback repression, arrows with no filling colour indicate activation.

Just like for L-methionine the production of L-lysine with *Corynebacterium glutamicum* is of great importance as food additive for animal feeding. It is possible to accumulate L-lysine in concentrations around 170 g/l in the fermentation process (Eggeling et al. 1999). In addition L-lysine belongs to the same group of amino acids as L-methionine, the aspartate family; both amino acids share intermediates for their own synthesis in metabolic pathways. The intermediate L-aspartate-β-semialdehyde (see Figure 2.7) is the branching point. L-Lysine overproducers possess strong activities of the enzyme dihydropicolinate synthase and reduced activities for homoserine dehydrogenase. If this ratio is contrary, the metabolic fluxes then lead to the accumulation of the intermediate homoserine and subsequently to the formation of L-

threonine and L-methionine. Also important is the inactivation of homoserine dehydrogenase in order to direct the metabolic flux to the L-lysine biochemical pathway. Another key enzyme is the aspartate kinase which catalyzes the reaction from L-aspartate to L-β-aspartyl phosphate. This enzyme is inhibited by both end products L-lysine and L-threonine, so it is important to break this inhibition process (Wittmann et al. 2004; Eggeling et al. 1999).

Figure 2.8 presents a comparison between the industrial processes to gain high purificated L-lysine hydrochloride on the one hand and the more economical process for the production of Biolys (developed by the Evonik company). The Biolys process is much cheaper and generates less liquid and solid waste but there are no purification steps. It is allowed to use the dried biomass of *Corynebacterium glutamicum* with high lysine concentrations as animal food additive. If it would be possible to establish L-methionine overproduction (the second important amino acid in animal food additives) with strains of *C. glutamicum*, in theory, the same industrial process could be used.

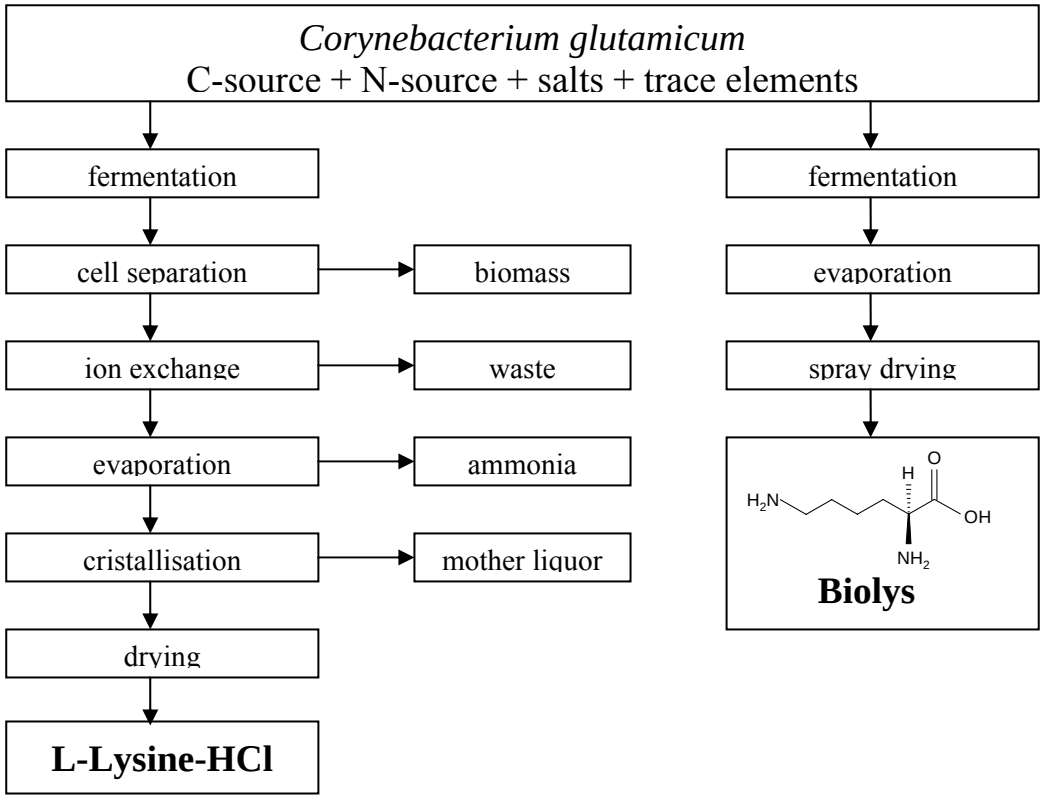


Figure 2.8: The Evonik (former Degussa) Biolys production scheme compared to the L-lysine-HCl (lysine hydrochloride) scheme (Leuchtenberger et al. 2005).

2.3.4 L-methionine production with *Corynebacterium glutamicum*

The first studies on microbial L-methionine production were performed in Japan in the 1970s (Kase et al. 1974; 1975). They used strains of *Corynebacterium glutamicum*. Also, the Japanese teams carried out the first research on the metabolic pathways of l-glutamic acid and l-methionine as well as the screening methods to develop new strains of *Corynebacterium glutamicum* (Shiio et al. 1980; Kase et al. 1975). The results on enzymes and precursors of L-methionine biosynthesis showed differences between this pathway in *Corynebacterium glutamicum* compared to other microorganisms. The greatest surprise was the development of the intermediate product O-acetylhomoserine (Figure 2.9). In most organisms another form of this product appears, O-succinylhomoserine. In Figure 2.9 it is shown that O-acetylhomoserine is an intermediate product for *Corynebacteria,* whereas O-succinylhomoserine is an intermediate product for *Escherichia coli* and *Pseudomonas aeruginosa*. In contrast *C. glutamicum* and *E.coli* share the formation of cystathione in the next step of biosynthesis, but in *P. aeruginosa* this precursor is omitted by direct catalysis to homocysteine, the last intermediate in L-methionine biosynthesis. Moreover, cystathione has the capability to use two alternative pathways leading to L-methionine synthesis: the *trans*-sulfuration pathway and the direct sulfhydrylation (Hwang et al. 2002; Lee et al. 2003). As shown in Figure 2.9 and also in Figure 2.10 in the *trans*-sulfuration pathway cystathione is incorporated as precursor and cysteine is metabolized. In the direct sulfhydrylation pathway cystathione is bypassed and instead of cysteine inorganic sulfur is used for metabolization. Whereas most organisms only have the ability to use one of these pathways, *Corynebacterium glutamicum* is flexible enough to utilize both. Each pathway is regulated by independent enzymes and tightly regulated by feedback repression.

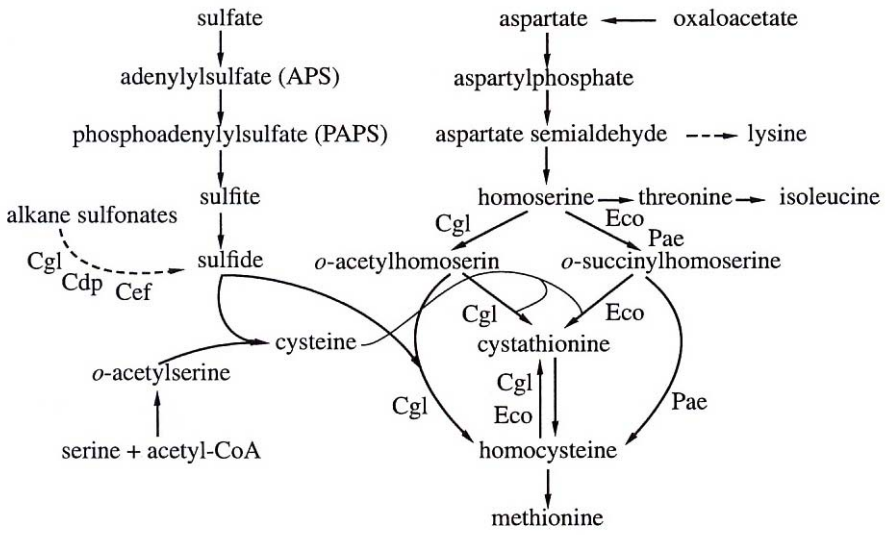
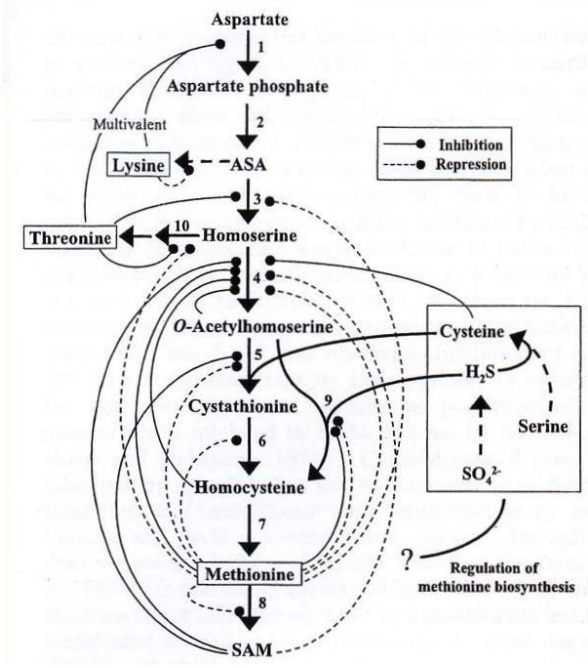


Figure 2.9: Regulation of L-methionine biosynthesis (Kovaleva 2007) in *Corynebacterium glutamicum* (Cgl), *Escherichia coli* (Eco) and *Pseudomonas aeruginosa* (Pae); Cdp (*Corynebacterium diphteriae*), Cef (*Corynebacterium efficiens*). The direct sulfhydrylation and *trans*-sulfuration pathways, which are alternatively used by different organisms, are indicated.

The regulation mechanisms and intermediates (feedback inhibition and repression) in *Corynebacterium* are different compared to other organisms, e.g. *Escherichia coli* (Kovaleva et al. 2007). In spite of the various regulation systems shown in Figure 2.10, it is easier to perform changed metabolic fluxes in this organisms than in others. This is due to the experiences researchers obtained in working with *Corynebacteria* strains to produce other amino acids. Several efforts have been made to characterize the L-methionine biosynthesis (Rückert et al. 2003; Kovaleva et al. 2007; Hwang et al. 2002).

In contrast to former investigations, where mostly the enzymes and activities were analyzed, recent studies contain detailed descriptions of the genes, which are responsible for the enzyme formation in L-methionine biosynthesis. Furthermore the possibilities to influence genes on transcription level by silencing and enhancing signals and control the amount and formation of key enzymes become increasingly dominant.

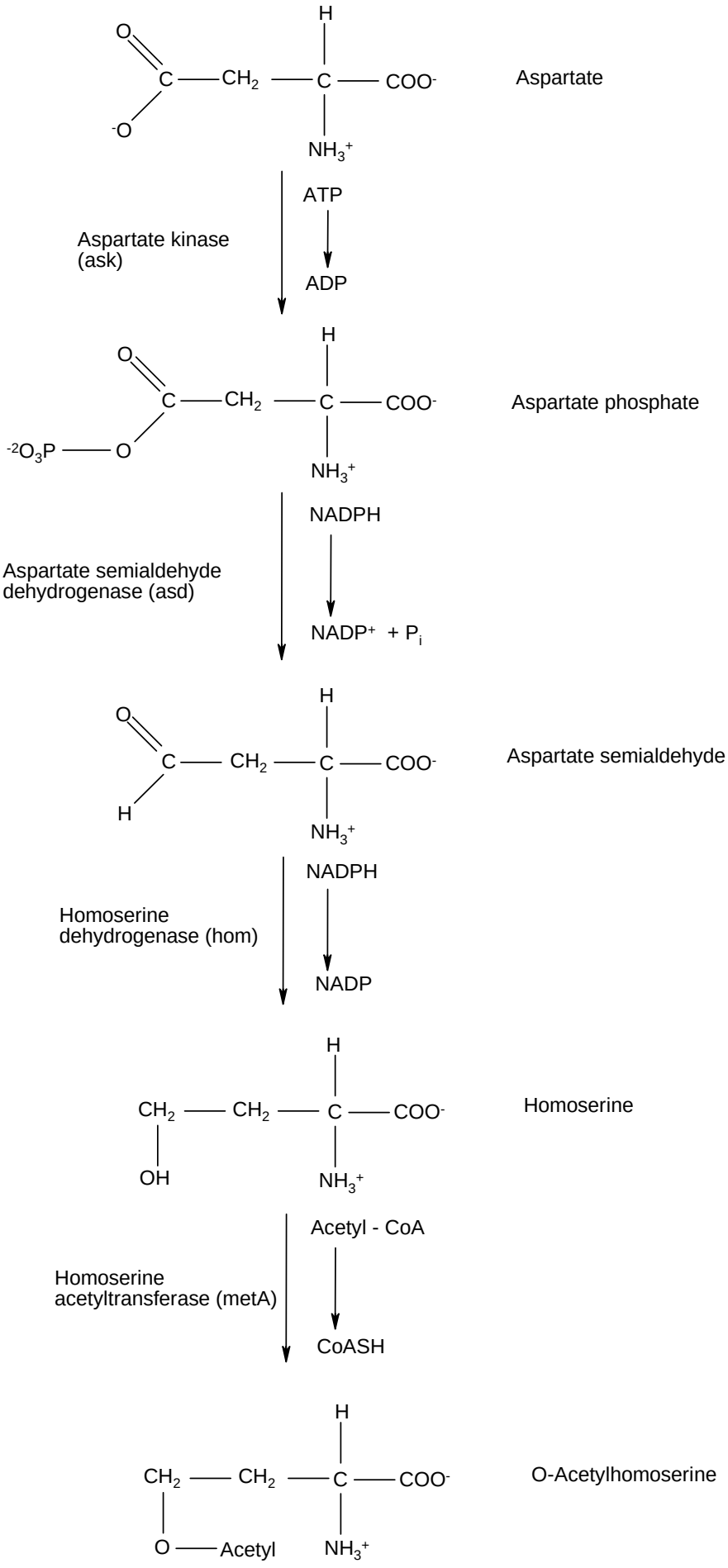


Enzymes in Fig.2.10 (see numbers)

1: aspartate kinase
2: aspartate semialdehyde
3: homoserine dehydrogenase
4: homoserine acetyltransferase
5: cystathione-γ-synthase
6: cystathione-β-lyase
7: homocysteine methylase
8: methionine adenosyl transferase
9: O-acetylhomoserine sulfhydrylase
10: homoserine kinase

Figure 2.10: Description of the signaling, inhibition and repression phenomenons of L-methionine biosynthesis in *Corynebacterium glutamicum*; dashed arrows indicate multiple steps. Straight and dashed lines with circular dots at the ends indicate inhibition and repression, respectively. Pathways of *trans*-sulfuration and direct sulfhydrylation are illustrated (Hwang et al. 2001).

The regulation mechanisms of L-methionine biosynthesis have been partially elucidated. The separation from L-lysine biosynthesis takes place with the conversion from aspartate semialdehyde to homoserine which is catalyzed by homoserine dehydrogenase. In the next step of synthesis the reaction to O-Acetylhomoserine means also the decoupling from the threonine pathway. From this point there is no sharing of precursors for other amino acid synthesis pathway any more. Figure 2.10 shows that the regulation of this catalysis by homoserine acetyltransferase is the highest regulated step in this metabolic pathway. There are 5 feedback inhibition and 2 feedback repression phenomenons influencing the enzyme activity or the gene transcription. The end products L-methionine and SAM (S-adenosyl-methionine), the bioactive form, have the capability to control the formation of several intermediates by inhibition or repression reactions.The chemical constitutional formulas of the biosynthesis of L-methionine are shown in Figure 2.11 to give an overview of the structures of precursors and reactions of chemical active groups important for the metabolic pathway of L-methionine. Additionally, the responsible genes for enzyme formation are mentioned (Rückert et al. 2003).


Aspartate
ATP
Aspartate kinase (ask)
ADP
Aspartate phosphate
NADPH
Aspartate semialdehyde dehydrogenase (asd)
NADP+ + Pi
Aspartate semialdehyde
NADPH
Homoserine dehydrogenase (hom)
NADP
Homoserine
Acetyl - CoA
Homoserine acetyltransferase (metA)
CoASH
O-Acetylhomoserine

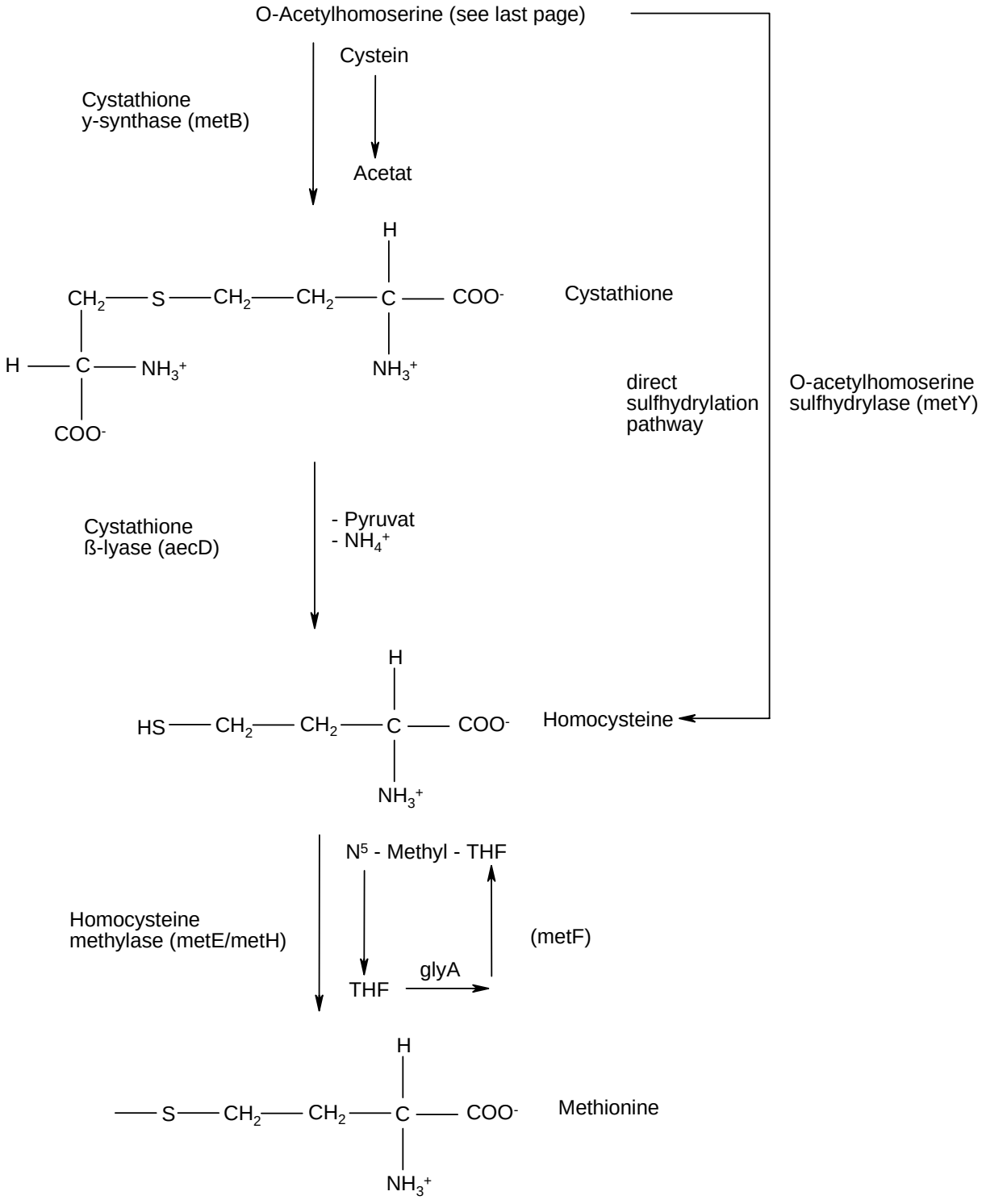


Figure 2.11: Pathway for the intermediates of L-methionine biosynthesis in *Corynebacterium glutamicum* in structural formulas; product names are written on the right, enzyme names on the left side, genes in brackets (modified after Voet et al. 2002 and Rückert et al. 2003).

Obvious in this context in Figure 2.11 is the existence of 2 genes (metE/metH) for the enzyme homocysteine methylase. MetE is a vitamin B_{12} independent, metH a vitamin B_{12} dependent methylase (Rückert et al. 2003).

In first studies on L-methionine production (Kase et al. 1974; 1975; Banik et al. 1974) the *Corynebacteria* strains were improved by random mutation and selection with methionine analogues / antimetabolites like ethionine. The strains were capable to produce approximately 2 g/l after 3 days.

In the 1990s research groups in India worked with auxotrophic mutants of *Corynebacteria* and other microbial strains on L-methionine overproduction (Mondal et al.1990; 1994; 1996). The strain improvement was also achieved by random mutations and L-methionine antimetabolites. L-methionine concentrations over 10 g/l in shake flask cultures could be reported.

In the last ten years more literature and information became available on L-methionine production with improved strains achieved by random mutation of *Corynebacterium glutamicum* (Sharma et al. 2001; Kumar et al. 2003). Concentrations of around 2.3 g/l of L-methionine were obtained according to these sources. In 2005 a comprehensive literature review of L-methionine producing strains was published (Gomes et al. 2005).

These and other studies also provide much information about cultivation conditions. The media composition for *Corynebacterium glutamicum* strains to gain optimal growth (Liebl et al. 1989) and effects of parameters like oxygen supply and pH to achieve higher production rates were described widely in the literature (e.g. Kumar et al. 2005).

The first study about attempts in genetic engineering of *Corynebacterium glutamicum* metabolism to overproduce L-methionine did not show high overproduction rates of L-methionine (Mampel et al. 2005), which means that a real break-through has not been achieved so far.

2.3.5 Amino acid excretion mechanisms in *Corynebacterium glutamicum*

The excretion of amino acids is an important factor for overproducing microorganisms. If the product is accumulated intracellularly, an additional step in down-stream processing is necessary, the cell disruption. For this reason the process would be much more expensive in industrial categories compared to product secretion in the extracellular environment. A high amount of accumulated substance in the cytosol has also other negative side effects. The production rate could be slowing down by cell signaling in order to avoid intracellular destruction.

For *Corynebacterium glutamicum*, which is a biotin deficient strain, the amount of this vitamin in minimal media is an important factor to get optimal excretion (Clement et al. 1986). According to this data it is easier for the organism to secrete L-glutamic acid while the biotin concentrations are in starvation conditions due to the incomplete formation of cell wall materials. In addition a temperature induced release of L-glutamic acid and its precise characterization has been reported recently (Stansen 2005). For L-glutamic acid there are reports about the active transport through the cell wall with a specific carrier system which has not been fully characterized and understood until now (Nakamura et al. 2007). Also there are characterizations of the **lysine exporter LysE** and the **threonine exporter ThrE** (Eggeling et al. 2003). For L-methionine connections between export and biotin concentration were observed (Mondal et al 1994).

Mechanisms for the general possibilities of the L-methionine export were described since the 90s (Krämer 1993). The export of amino acids is based in most cases on a secondary transport system. New results about the export system for L-methionine were published in 2001 and 2005 (Eggeling et al. 2001; Trötschel et al. 2005). According to this data the L-methionine export systems are tightly regulated. The overexpression of distinct membrane proteins is linked with increased intracellular concentrations of methionine. One responsible **gene**, **BrnF**, **coding for the larger subunit of BrnFE**, has been identified as being responsible for one **methionine export system** (and furthermore also for isoleucine export). Additionally, strong indications for the presence of at least one other export system, presumably with low affinity but high capacity, exist due to the investigations in the works of Eggeling (2001) and Trötschel (2005).

2.4 Biochemical amino acid degradation

Apart from sugars, amino acids are common substrates for microbial cell growth. Sources of amino acids, like peptones, tryptones and casamino acids are used in laboratory media. The degradation of individual amino acids depends on the availability of other energy sources and on the C/N ratio.

The first step in biochemical amino acid degradation is, in general, the removal of the α-amino acid group. The reaction products are 2-oxo acids. There are different biochemical ways to perform this reaction (Lengeler et al. 1999). They are, as follows:

- the oxidative deamination
- the transamination
- the β-elimination

After the α-amino group removal further degradation steps take place according to the backbones of the 2-oxo acids (aliphatic, heterocyclic, aromatic compounds).

The biochemical degradation products of amino acids are normally intermediates of the citric acid cycle or precursors of these intermediates. They are converted to carbon dioxide and water or will be applied in the gluconeogenesis. The degradation of methionine and other amino acids which are important in this thesis is shown in Figure 2.12. The amino acids can be divided in two groups of catabolic degradation, the glucogenic and the ketogenic amino acids. The ketogenic amino acids are converted to acetyl-CoA or acetoacetate. Afterwards they are transformed to fatty acids or ketone bodies. Lysine, leucine and threonine belong to this group of amino acids which can be degraded only this way. The degradation products of these three amino acids cannot be used for gluconeogenesis. Tryptophane, tyrosine, iso-leucine and phenylalanine are amino acids which can be metabolized on both biochemical ways for degradation, the ketogenic and the glucogenic one. The other amino acid groups, shown in Figure 2.12, are degraded through the glucogenic ways. This means that their degradation products are available for gluconeogenesis. Glucose precursors like pyruvate, α-ketoglutarate and succinyl-CoA are synthesized on these biochemical pathways. Methionine is converted to succinyl-CoA, glutamic acid to α-ketoglutarate and alanine as well as glycine are degraded through transamination to pyruvate and following acetyl-CoA. The biochemical degradation

is an important explanation approach to discuss increased and decreased amino acid concentrations under nutrient deficient condition phases in shake flask and bioreactor cultivations. Under those conditions the microorganisms try to find possibilities to maintain the metabolism by degradation or conversion of available substances, like e.g. the conversion of amino acids (Voet et al. 2002).

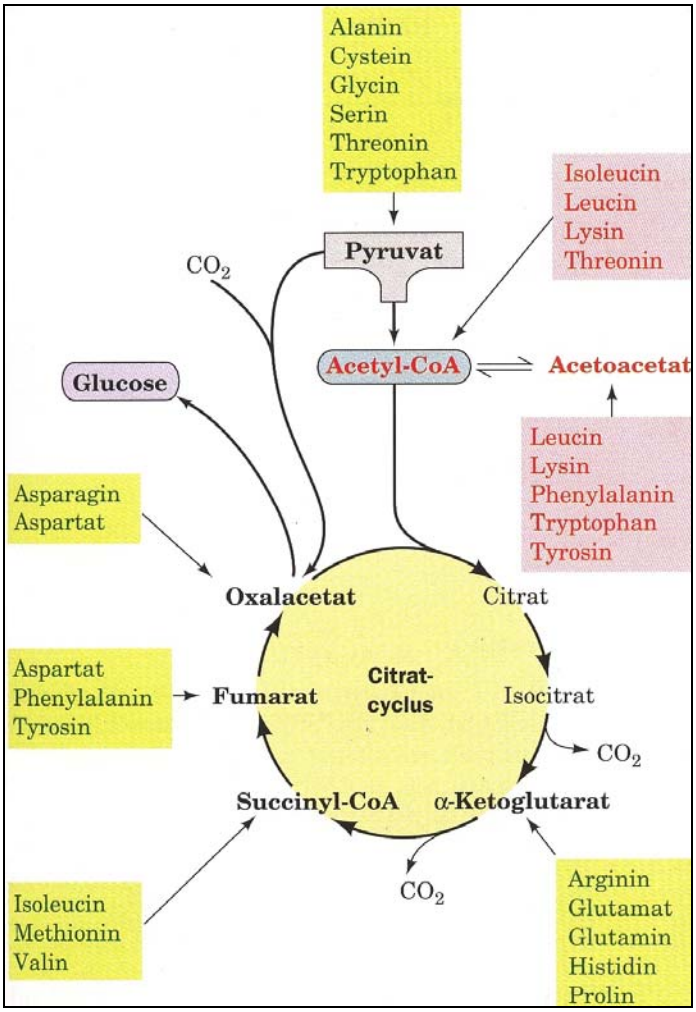


Figure 2.12: Biochemical amino acid degradation to intermediates of the citric acid cycle. The glucogenic degradation is coloured in green, the ketogenic one in red / violet (Voet et al. 2002).

2.5 Random mutagenesis and following selection

Random mutation is a basis method in biochemistry to improve the abilities of microorganisms. Mutation is performed by the use of highly potent chemical mutagens for bacteria like NTG (N-methyl-N'-nitro-nitrosoguanidine) and EMS (ethyl-methanesulfonate) or ultraviolet (UV) radiation. NTG induces primarily base transition mutations of the GC→ AT type, although AT→GC transitions, transversions, and even frameshifts appeared at low frequencies (Gerhardt et al. 1981). EMS works in similar ways, both NTG and EMS are also dangerous carcinogens! The ultraviolet radiation of microorganisms does not have the same potential in mutation compared to EMS and NTG; working with this method is, however, much less dangerous. Therefore, the handling of UV radiation in screening systems is far easier and the process is much more ecological because due to the natural occurrence of UV radiation an environment which influences all living organisms on earth. Usually UV rays around 250 nm are deployed in experiments.

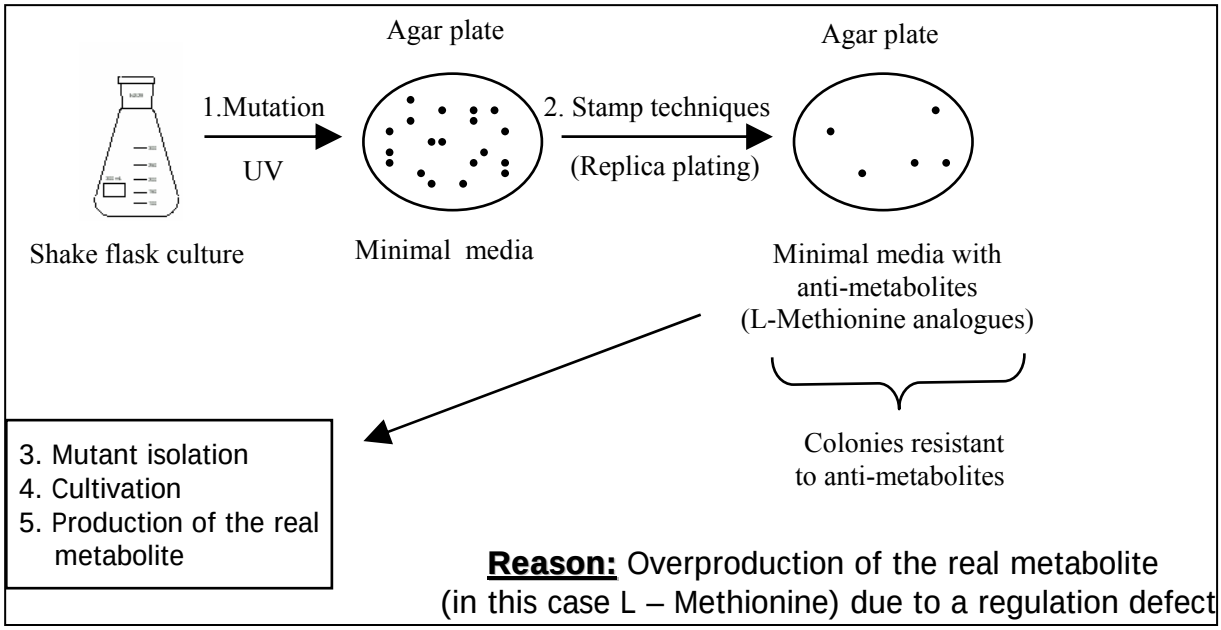


Figure 2.13: Concept for random mutation and the succeeding selection method to get overproducers for the amino acid L-methionine.

The selection method is crucial because the multitude of obtained microorganisms has to be reduced for analysis. In Figure 2.13 the principle method for the random mutation and the following selection step is documented for the case of L-methionine. In this example the selection is performed by replica plating (a wide-spread microbiological method) on agar plates

with minimal media and, in addition, L-methionine analogues / anti-metabolites. These anti-metabolites are almost molecular structural analogues to L-methionine. In theory all microorganisms with functional feedback and inhibition signaling systems do not produce L-methionine in the presence of high L-methionine concentrations in media but metabolize the available supply. If the microorganisms mix up methionine with the L-methionine analogues and deactivate their production pathways, then microbial death is unevitable. It is not possible to utilize analogues of amino acids for metabolism. On the other hand mutant strains derived from UV radiation with deactivated signaling sytems for distinct feedback inhibiton and repression systems are able to produce L-methionine in high amounts. These mutants which are resistant to methionine analogues have altered and deregulated enzymes that are not sensitive to feedback inhibition or repression due to mutations in structural important genes for this signalling system (Kumar et al. 2005).

There are various structural analogues of L-methionine described in the literature like ethionine, norleucine, methyl methionine, methionine sulfoxide, methionine methylsulfonium chloride and others. The difference of the constitutional formula of L-methionine compared to the structural analogues L-ethionine and L-norleucine is shown in Figure 2.14. L-Ethionine has a CH_2-group (methylene) more as L-methionine, L-norleucine does not possess a sulphur component. The usage of ethionine is mentioned as the most effective substance (Mondal et al. 1994. Kumar et al. 2003). Nevertheless the development of fast and effective analysis systems is absolutely required to enhance the chances to obtain mutant strains with improved properties.

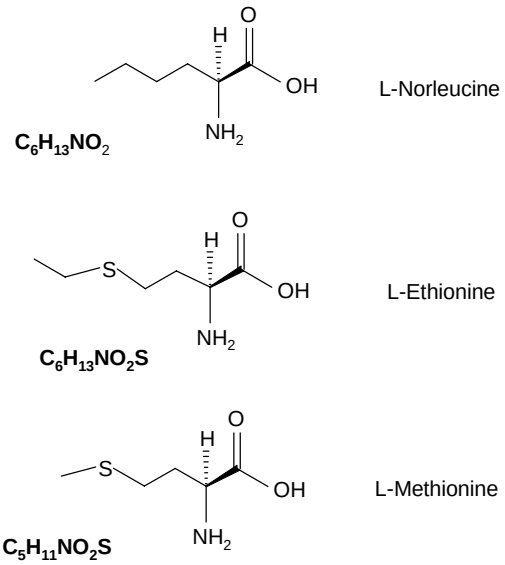


Figure 2.14: Comparison of the chemical structure of the amino acid L-methionine and the structural analogues / anti-metabolites L-ethionine and L-norleucine.

2.6 Amino Acid Analysis

The introduction and refinement in analysis of amino acids, especially L-methionine, is another important point to get reproducible results, particularly with regard to the huge amounts of samples achieved by the screening.

Various methods for the analysis of amino acids are mentioned in the literature. In former investigations it was common to separate amino acid mixtures with paper chromatography, thin layer chromatography (TLC) or ion exchange chromatography (IEC). Afterwards, the analysis was performed by ninhydrin reaction (Kase et al. 1974). In presence of ninhydrine the heating of amino acids leads to an oxidative desaminination und at the same time a decarboxylation of the amino acids. This results in formation of carbon dioxide and an aldehyde which is one C-atom shortened. For quantitative acquisition of data blue violet dye (Ruhemann's violet), formed in a following reaction, has been used (Stahl 1967). Today the detection systems for amino acids are high pressure liquid / performance chromatography (HPLC) and gas chromatography (GC) (Husek et al. 2001).

Many studies are available about a derivatization of amino acids before separation in order to make the following detection easier. On the basis of Edman's degradation of proteins (Voet et al. 2002) with phenylisothiocyanate (PITC) to obtain the amino acid sequence, the analysis of amino acid mixtures by derivatization with PITC and detection with HPLC is widely described (e.g. Heinrikson et al. 1984). One of the state-of-the-art techniques is the derivatization with ortho-phtaldialdehyde (OPA). This reaction is distinguished by high sensitivity but a problem is the missing reaction with secondary amines. An alternative is e.g. the reaction with 6-amino-quinolyl-N-hydroxy-succinimidyl-carbamate (ACQ). (Naidanow et al. 2005). Today a broad range of derivatization agents for amino acid detection are available. It is common to separate the amino acids with reversed phase chromatography, which means that the stationary phase (column solid material) is unpolar (e.g. by reaction of silica gel with silanes) and the mobile phase (the eluent) is polar (Heinrikson et al. 1984 + Husek et al. 2001). Special chiral colums for separation and detection of enantiomeric forms of amino acids in HPLC and GC are developed. The combination sytem of GC / HPLC with mass spectrometry (MS) allows to get a molecular fingerprint of each chemical compound which is an important tool for reliable identification of substances.

Due to problems with derivatization and in order to accelerate the analysis, attempts to detect amino acids in unmodified conditions with commercial liquid detectors have been made (Petritis et al. 2002). In comparison with several detector types, the chemiluminescent nitrogen detection (CLND) and the tandem mass spectrometry (MS) show high sensitivity and specificity.

3 Materials and methods

3.1 Microbial strains

Arthrobacter aurescens DSM7330

This bacteria also belongs to the group of *coryne*-form bacteria. It was isolated from soil samples in screening experiments to convert aryl hydantoins (Voelkel 1993). The physiological and morphological properties are the positive gram staining, strictly aerobic, pleomorphic growth and the amotility. The organism is nonsporulating. In this research the strain was used to demonstrate the efficiency of obtaining L-methionine in a biocatalysis experiment. It is the target to show the broad spectrum of possibilities to gain amino acids like L-methionine.

Corynebacterium glutamicum DSM20300 / ATCC13032

This is the most common *C. glutamicum* strain for investigations. In 1957 the soil bacterium was isolated under the name *Micrococcus glutamicus* (e.g. Kinoshita et al. 1957). The ability to produce L-glutamic acid was described widely in the literature (Kinoshita et al. 1961). It is the base material for all research on amino acid overproduction and is available at all famous cell culture collections like DSM (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Germany) and ATCC (American type culture collection). In this study the strain has been used to reproduce the L-glutamic acid production and in random mutagenesis in order to get new overproducers of L-methionine.

Corynebacterium glutamicum ATCC21608

This strain is commercially available at the ATCC. According to information (Nakayama et al. 1973) the microorganism might be able to produce L-methionine. It was obtained by random mutation with methyl methionine as structural analogue/anti-metabolite. The base material for the experiments was the strain *C. glutamicum* DSM20300 / ATCC13032.

Corynebacterium glutamicum KY10574

This strain was also received by random mutation of *C. glutamicum* DSM20300 / ATCC13032 to improve the L-methionine production abilities. It was directly delivered by the Japanese company Kyowa Hakko Kogyo Ltd, Tokyo. It is the most important strain for this study, several experiments described in this work were carried out with these bacteria in order to optimize the production rates and to get information about adequate physico-chemical and biochemical parameters for cultivation. In the picture in Figure 3.1 (recorded with an electron microscop) the rod-shaped form of the bacterial species *Corynebacterium glutamicum* is shown in many examples. The coryne-form of the cells is not that clear to see due to the fact, that *Corynebacterium* is a pleiomorphic (form variability) species (Lengeler et al. 1999). Packages of several cells in parallel arrangement ("palisades") can also be observed.

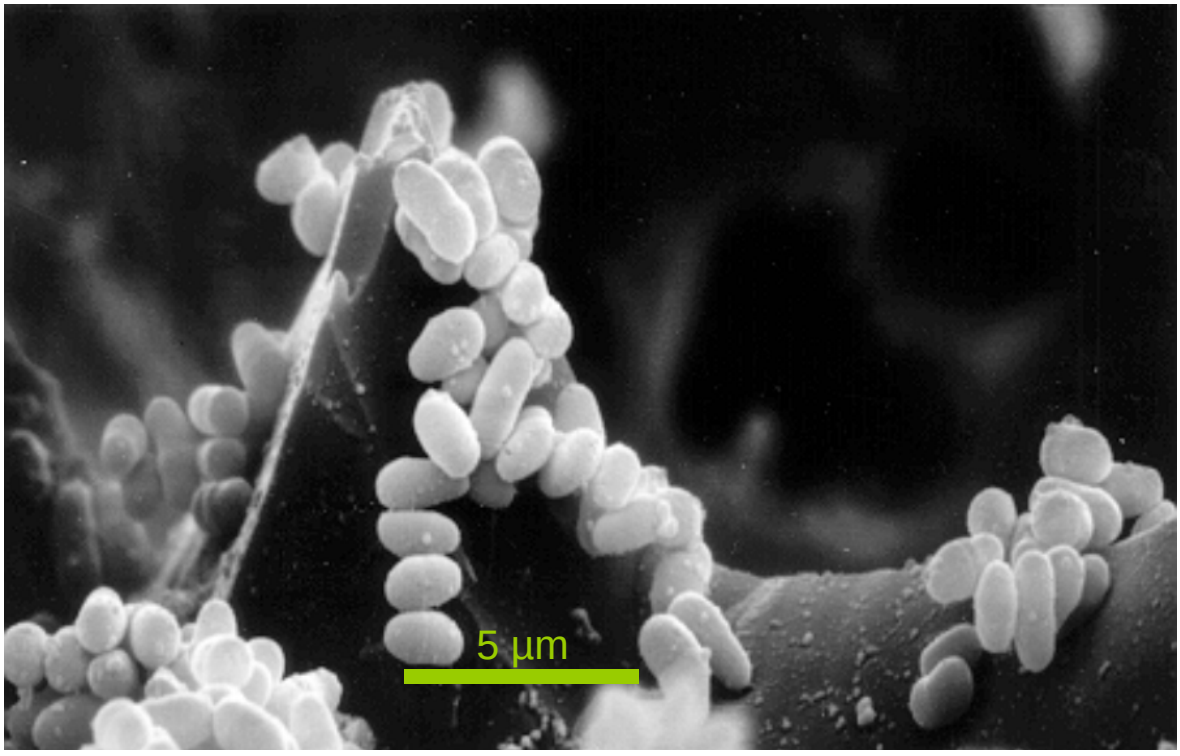


Figure 3.1: Electron microscopic picture of *Corynebacterium glutamicum* cells, recorded at the Forschungszentrum Jülich, Institute of Biotechnology (IBT).

All bacteria of the species *Corynebacterium glutamicum* possess the GRAS status (generally regarded as safe). For general information about universal *Corynebacterium glutamicum* characteristics, see chapter 2.3.1.

3.2 Media and biotransformation experiments with *Arthrobacter aurescens* DSM7330

The experiments with this strain were specifically described in several PhD - studies from the 1990s which had been undertaken at the Institute for Biochemistry and Biotechnology, TU Braunschweig (Voelkel 1993; Stehr 1996). Therefore, access to these microbial material was easy to get. Thus, there will be only a short summary of media composition and the way to perform the biocatalysis experiments in this research (for further information please have a look in the PhD theses from Voelkel and Stehr).

The following information about media compositions and biocatalysis are based on the studies from Stehr (1996).

The first step of the experiments was the microbial growth of *Arthrobacter aurescens* to gain biomass and to activate the enzyme acitivities using the inducer DL-N-3-methyl-5-indolyl-methyl-hydantoin (IMH). The biomass was centrifuged and afterwards employed in the biotransformation investigations.

The biotransformation was performed in 100 ml shake flasks (each with 2 baffles). The vessels were pre-filled with 9 ml substrate solution (0.1 molar glycine / NaOH buffer, 5.7 mM DL-MTEH (methyl-thio-ethyl-hydantoin)). After that 1 ml of wet biomass in a concentration of 50 g/l was added. The biotransformation reaction was carried out in a water bath vessel for 24 h at 37°C. The initial pH of 8.5 was controlled and adjusted correctly in the first 5 hours. The samples were centrifuged (Heraeus Christ Biofuge A) for 15 min at 13,000 rpm and stored at 4°C in the refrigerator. The supernatant was analysed with HPLC (high pressure liquid chromatography).

3.3 *Corynebacterium glutamicum* media

3.3.1 Conservation media

The conservation of the *Corynebacteria* strain material was carried out using a complex BY (bouillon yeast extract) medium (see table 3.1):
After inoculation 50 ml BY medium in a 250 ml shake flask (2 baffles) was cultivated for one day (parameters: 30°C, 120 rpm). Afterwards, for short time conservation BY agar media plates were inoculated with 50 µl and also cultivated for one day. These agar plates were stored at 4°C in the refrigerator. They form the basis material for all experiments. The long time conservation was carried out with glycerine stocks. 1 ml of a bacterial sample was mixed with 1 ml glycerine in plastic tubes and stored at -20°C.

Table 3.1: Composition of complex **BY** (agar) medium for strain conservation.

20 g	standard-I-nutrition bouillon
5 g	yeast extract
22 g	agar-agar (only for solid plate media)
→	pH 7.2 adjusted with 6N NaOH
→	fill up to 1,000 ml with distilled H_2O; sterilization

3.3.2 Media for *Corynebacterium glutamicum* DSM20300

There were two minimal media in use for the strain *Corynebacterium glutamicum* DSM20300, one for the screening after L-methionine overproducers, one for the reproduction of L-glutamic acid production. The minimal medium for the screening system was approximated to L-lysine production media CGXII with some modifications (Keilhauer et al. 1993; Deutenberg 2003).

Table 3.2: Composition of screening minimal medium **SMM**

Solution 1: basic components

20 g	$(NH_4)_2SO_4$
10 g	$(NH_4)_2S_2O_3$
5 g	urea
1 g	KH_2PO_4
2 g	K_2HPO_4
0.25 g	$MgSO_4 \cdot 7\ H_2O$
0.3 mg	3,4 - dihydroxy benzoic acid
→	fill up to 894 ml with distilled H_2O
→	pH 7.2 adjusted with 2N NaOH
→	sterilisation

Solution 2: glucose

50 g	glucose
→	fill up to 100 ml with distilled H_2O
→	sterilisation

Solution 3: mineral elements

200 mg	$CaCl_2$
200 mg	$FeSO_4 \cdot 7\ H_2O$
200 mg	$MnSO_4 \cdot H_2O$
20 mg	$ZnSO_4 \cdot 7\ H_2O$
6 mg	3,4 - dihydroxy benzoic acid
1.2 mg	$CuSO_4$
200 µg	$NiCl_2 \cdot 6\ H_2O$
→	fill up to 1,000 ml with distilled H_2O
→	sterile filtration

Solution 4: vitamin stock solution

200 mg	biotin
200 mg	thiamin hydrochloride
→	fill up to 1,000 ml with distilled H_2O
→	sterile filtration

Composition of **SMM** medium:

894 ml solution 1, 100 ml solution 2, 5 ml solution 3 and 1 ml solution 4 were pooled. The medium SMM was used in several experiments in different scales. For the screening experiments in 96 – well plates amounts of 250 µl medium per well were used. The medium volume in the shake flask and bioreactor scales contained up to 3.5 litres. The temperature in all experiments was 30°C, in the shake flask experiments shaking rates of 120 rpm were used.

For the production of L-glutamic acid the media were made according to instructions from the original patent sources (Kinoshita et al. 1961).

Table 3.3: Composition of media for L-glutamic acid production

Complex preculture for L-glutamic acid production **CP**

10 g	peptone, tryptic
10 g	yeast extract
5 g	glucose
5 g	NaCl
→	fill up to 1,000 ml with distilled H_2O
→	pH 7.2 adjusted with 2N NaOH and 2 molar HCl
→	sterilisation

Minimal production medium for L-glutamic acid production **MPM**

5 g	urea
0.1 g	$MgSO_4 \cdot 7\ h_2O$
0.5 g	K_2HPO_4
0.005 g	$FeCl_3 \cdot 6\ H_2O$
→	fill up to 900 ml with distilled H_2O
→	pH 7.5 adjusted with 2N NaOH and 2 molar HCl
→	sterilisation
→	addition of separately sterilised glucose solution (50g / 100 ml)

The precultivation was performed in a 500 ml shake flask with 2 baffles with 100 ml of CP medium at a temperature of 30°C and 100 rpm for 17 h. The inoculation was done directly with a loop of cell material from agar plates (BY medium, see chapter 3.3.1). The main culture was inoculated with 2 ml of the incubated preculture on 100 ml of minimal production medium MPM (see Table 3.3). Afterwards, the cultivation was performed under the following conditions: 144 h, 30°C, 100 rpm, 102 ml working volume in a 500 ml shake flask (2 baffles).

3.3.3 Media for *Corynebacterium glutamicum* ATCC21608

The strain *Corynebacterium glutamicum* ATCC21608 was investigated using the same media as for the strain *Corynebacterium glutamicum* KY10574. For further information about these media (KH2 / KH1 / F1) see chapter 3.3.4.

3.3.4 Media for *Corynebacterium glutamicum* KY10574

The first cultivation instructions (media KH1 und KH2, see Tables 3.4 and 3.5) for the strain *C. glutamicum* KY10574 were obtained from Kyowa Hakko Kogyo Ltd. The complex media KH1 was directly inoculated from an agar plate, after 24 h cultivation time (parameters: 30°C, 120 rpm, 25 ml medium in a 250 ml shake flask (2 baffles) the production media KH2 was inoculated in a 1:11 ratio.

Table 3.4: Composition of complex seed medium **KH1**(Kyowa Hakko)

10 g	peptone, tryptic
10 g	yeast extract
2.5 g	NaCl
→	pH 7.4 adjusted with 6N NaOH
→	fill up to 800 ml with distilled H_2O; sterilization
→	addition of separately sterilised glucose solution (20 g/200 ml)

The production medium KH2 (see Table 3.5) is cloudy due to the very unsoluble $CaCO_3$ component. The cultivations were performed in 11 ml scale in a 250 ml shake flask with 2 baffles (1 ml KH1 + 10 ml KH2) and 110 ml scale in a 1 L shake flask (10 ml KH1 + 100 ml KH2) for 4 days. The experiments were carried out at 30°C and 120 rpm.

Table 3.5: Composition of production minimal medium **KH2** (Kyowa Hakko)

20 g	$(NH_4)_2SO_4$
0.5 g	K_2HPO_4
0.5 g	KH_2PO_4
1 g	$MgSO_4 \cdot 7\ H_2O$
100 mg	$FeSO_4 \cdot 7\ H_2O$
100 mg	$MnSO_4 \cdot 4\ H_2O$
20 g	$CaCO_3$
→	pH 7.4 adjusted with 6N NaOH
→	fill up to 699 ml with distilled H_2O; sterilization
→	addition of separately sterilised glucose solution (100 g/300 ml)
→	sterile filtration: 1 ml biotin (from 100 mg/l stock solution)

In cooperation with the vTi Braunschweig (Johann Heinrich von Thünen Institute, Institute of Agricultural Technology) and after an extensive literature search concerning *Corynebacterium glutamicum* a minimal medium (F1 medium, see Table 3.6) has been developed in order to get bacterial growth without the influence of complex media components. The medium was produced through mixing of several solutions (see Table 3.6).

The mixing ratios of the liquids were as follows: 200 ml solution A, 100 ml solution B, 5 ml stock solution C, 300 ml solution D, 5 ml stock solution E, 2 ml stock solution F, 350 ml solution G, 5 ml solution H, 10 ml stock solution I, 10 ml stock solution J. Afterwards, the liquid amounted to 987 ml.

5 ml F1 medium were taken out as aliquote to adjust pH to 7.3 with NaOH, the appropriate amount of NaOH was added to the medium, last of all the medium was filled up with H_2O to 1000 ml.

In order to get information about influence of carbon source on amino acid production (especially L-methionine), the glucose concentration was altered in the following concentration ranges between 20 and 100 g/l.

As well as for the KH-media described before the cultivations were performed in comparable scales. In a 250 ml shake flask with 3 baffles a working volume of 25 ml was used, in a 1 L shake flask 100 ml working volume was utilized. The general parameters were also 30°C for the temperature; the shaking speed varied between 120 and 210 rpm.

Table 3.6: Composition of **F1** minimal medium

Solution A	20 g	glucose
	→	fill up to 200 ml with distilled H_2O; sterilization

Solution B	30 g	KH_2PO_4
	60 g	K_2HPO_4
	5 g	NaCl
	→	pH 7.3 adjusted with 6N NaOH
	→	fill up to 1,000 ml with distilled H_2O; sterilization

Solution C	6 g	$MgCl_2$
	2.5 g	$CaCl_2 \cdot 2\ H_2O$
	→	fill up to 1,000 ml with distilled H_2O; sterile filtration

Solution D	10 g	$(NH_4)_2SO_4$
	→	fill up to 300 ml with distilled H_2O; sterilization

Solution E	4 g	$FeSO_4 \cdot 7\ H_2O$
	50 g	$MgSO_4 \cdot 7\ H_2O$
	1 g	$MnSO_4 \cdot H_2O$
	1 g	NaCl
	→	fill up to 1,000 ml with 0.1 molar HCl; sterile filtration

Solution F	100 mg	$Na_2B_4O_7 \cdot 10\ H_2O$
	50 mg	$(NH_4)_6MoO_{24} \cdot 4\ H_2O$
	800 mg	$ZnSO_4 \cdot 7\ H_2O$
	270 mg	$CuSO_4 \cdot 5\ H_2O$
	7.5 mg	$MnCl_2 \cdot 4\ H_2O$
	870 mg	$FeCl_3 \cdot 6\ H_2O$
	10 mg	$NiCl_2 \cdot 6\ H_2O$
	→	fill up to 1,000 ml with 0.05 molar HCl; sterile filtration

Solution G	5 g	urea
	→	fill up to 350 ml with distilled H_2O; sterilization

Solution H	3 g	3,4 - dihydroxy benzoic acid
	→	fill up to 350 ml with distilled H_2O; sterile filtration

Solution I	100 mg	biotin
	→	fill up to 1,000 ml with distilled H_2O; sterile filtration

Solution J	20 mg	thiamin hydrochloride
	20 mg	cyanocobalamin
	→	fill up to 1,000 ml with distilled H_2O; sterile filtration

3.4 Analysis techniques

3.4.1 Bio dry mass and optical density

The bio dry mass was measured using metal tubes with 15 ml liquid samples and small plastic reaction vials (Eppendorff caps) with 1 ml liquid samples. The small vials were used due to the limitation of the amount of liquid in small scale cultivations in shake flasks. The measurement of a single sample was always performed twice to sure the accuracy. The samples were centrifuged for 15 minutes at 12,000 rpm (Heraeus Christ Biofuge A for 1.5 ml vials; Heraeus Sepatech Biofuge 17RS for metal tubes), the supernatants were discarded. The vessels were stored in a heater (100°C) for 24 h, afterwards the bio dry mass in the vessels was measured against the empty weight. The samples with the nearly unsoluble $CaCO_3$ medium component were measured by giving 200 µl of 4 molar hydrochloric acid to a volume of 800 µl sample liquid in order to dissolve the $CaCO_3$ component.

The optical density was measured photometrically at a wavelength of 546 nm (photometer type: Ultrospec 3000; Pharmacia Biotech). The samples were diluted in a ratio of 1:100 in order to keep the absorption level in the linear area. The blank sample was the sterilised medium which was also diluted in a ratio of 1:100.

3.4.1.1 Connection between the BDM and OD_{546} values

The connection between the measurement of bio dry mass (BDM) and the optical density at 546 nm (OD_{546}) in F1 minimal medium is presented in Figure 3.2. Both measurements are indicators for cell growth of the *C. glutamicum* microorganisms. The values which were obtained from the measurement of both parameters in the shake flask cultivations as described in chapter 4.4.3 were plotted in this graph. The development of the curves for both parameters was nearly parallel in almost all cases shown in this chapter (see Figures 4.19 – 4.24).

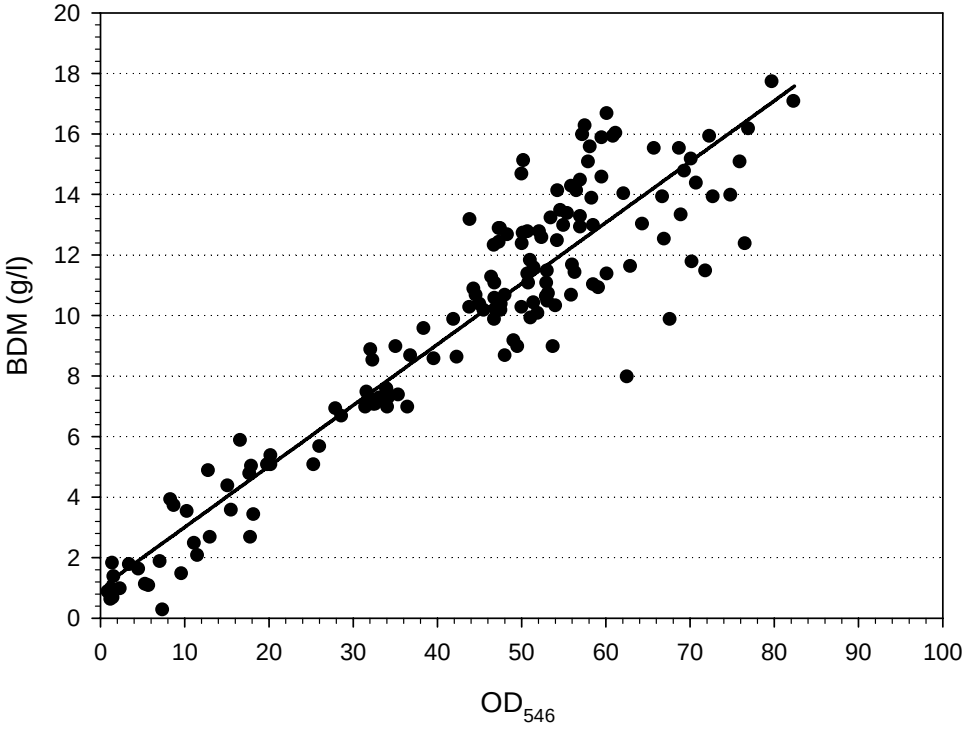


Figure 3.2: Connection between the parameters for the cell growth of *C.glutamicum* KY10574 in F1 minimal medium, the plotted values were obtained from the shake flask cultivations described in chapter 4.4.3.

3.4.2 pH-measurement

The pH measurement was done potentiometrically with a pH-electrode type Schott CG 802. For calibration 2 control solutions (pH 4.01 and 7.00) were used.

3.4.3 Glucose measurement

The glucose measurement was performed with an enzymatic photometric test system (photometer type: Ultrospec 3000; Pharmacia Biotech) for D-glucose, by the companies Boehringer Mannheim / R-Biopharm. The detection based on UV-absorption at 365 nm.

The test system contains three liquid chemicals in bottles:

- The first solution (now named **solution 1**) consists of 7.2 g powder mixture, which has to be dissolved in 45 ml H_2O. According to the producer this **solution 1** contains triethanolamine buffer (pH approx. 7.6), NADP (approx. 110 mg), ATP (approx. 260 mg) and magnesium sulfate.
- The second liquid chemical is a suspension (now named **suspension 2**) consisting of the enzymes hexokinase (approx. 320 Units) and glucose-6-phosphate dehydrogenase (approx. 160 Units).
- Solution 3 contains a standard solution of D-glucose for assay control purposes.

The reagents were directly given and mixed in plastic cuvettes suitable for photometric purposes, the final volume was 3.020 ml. The reaction has to be carried out at a temperature of 20 – 25°C. A schematic plan of the procedure is presented in Table 3.7.

Table 3.7: Schematic plan of the substance mixing for D-glucose measurement (Enzymatic test system Boehringer Mannheim / R-Biopharm)

pipette into cuvette	blank	sample
solution 1	1,000 ml	1,000 ml
sample	-	0.1 ml
H_2O, dest.	2,000 ml	1,900 ml
→ mix and read absorbances of the solutions (A_1) after approx. 3 min.		
→ the enzymatic reaction is started by addition of:		
suspension 2	0.02 ml	0.02 ml
→ mix and read absorbances (A_2) after the reaction has stopped (approx. 10 – 15 min)		

The reaction mechanism of this D-glucose measurement system is based on two enzymatic reactions:

$$\text{D-glucose + ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate + ADP}$$

$$\text{glucose-6-phosphate + } NADP^+ \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-phosphate + } NADPH^+ + H^+$$

The amount of $NADPH^+$ formed in this reaction is stoichiometric to the amount of D-glucose. The increase in NADPH is measured by means of its light absorbance at 365 nm.

The general equation to calculate the concentration is:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A_{D-glucose}$$

c = concentration of D-glucose (g/l), V = final volume (ml), v = sample volume (ml), MW = molecular weight of the substance to be assayed (g/mol, 180.16 g/mol for D-glucose); d = light path (cm), ε = extinction coefficient of NADPH at 365 nm =3.5 (L • mmol • cm^{-1}), ΔA = Absorbance differences (A_2-A_1, see Table 3.7)

For D-glucose:

$$c = \frac{3.020 \times 180.16}{\varepsilon \times d \times v \times 1000} \times \Delta A_{D-glucose} = \frac{5.441}{\varepsilon} \times \Delta A_{D-glucose}$$

If samples were diluted, the result had to be multiplied by the dilution factor (for further information about the details see reference material of Boehringer Mannheim / R-Biopharm).

3.4.4 Thin layer chromatography (TLC)

The thin layer chromatography (TLC) is a quick and cheap method to detect amino acids in a qualitative way. The samples dissolve in an appropriate solvent (mobile phase) and are drawn up on the solid stationary phase via capillary action. The amino acids are separated according to their polarities and the strength of their interactions with the stationary phase. The detection is based on a dye producing reaction of the amino acids with ninhydrin.

For the experiments in this work a thin layer of silica gel (Silica gel 60 $F_{254.}$ Merck KGaA) immobilised on aluminium sheets was used as stationary phase. The samples were placed on the aluminium sheets in an amount of 5 µl with a micro pipette. After drying, the aluminium sheets were placed in a closed glass chamber with the mobile phase which covered the chamber ground (fill level: 1 cm). The mobile phase was a solution mixed of n-butanol / glacial acetic acid / H_2O in a ratio of 12:3:5 (v/v/v). It was important to prevent immediate contact between the solvents (mobile phase) and the samples because the amino acid spots then were very clear defined. After chromatography the aluminium sheet was dried with a heater, then the surface of the sheet was sprayed with ninhydrin reagent. Finally the sheet was dried once again on the heater.

The thin layer chromatography of the biotransformation samples was performed using a chiral TLC plate (Macherey-Nagel company, chiral plate coated with Cu^{2+} and chiral reagent), which was capable of separating DL enantiomeric product forms. The mobile phase in this case consisted of a mixture of methanol / H_2O / acetonitrile in a ratio of 50:50:200 (v/v/v). The other working stages and the ninhydrin detection were carried out in the same manner as for the other TLC experiments.

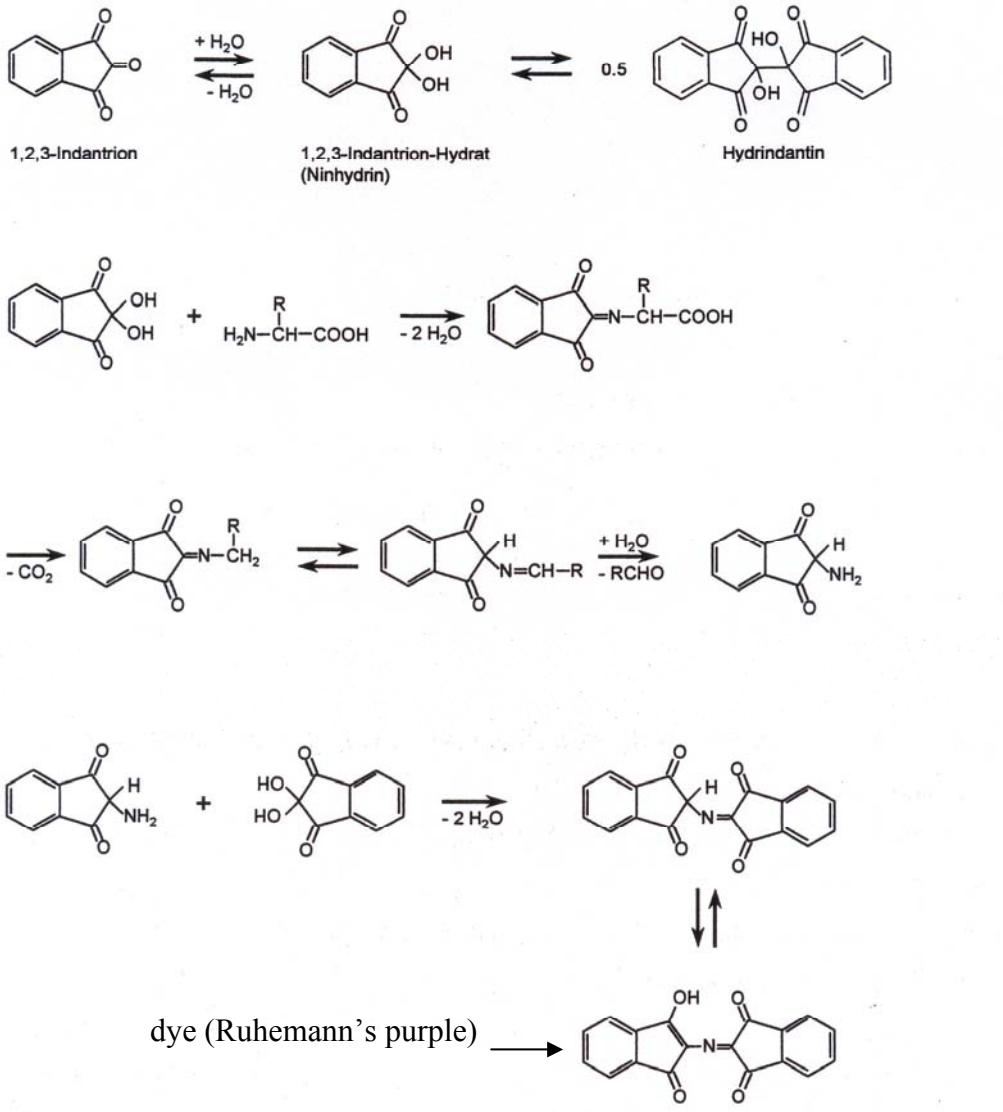


Figure 3.3: Reaction of ninhydrin with amino acids (Stahl 1967)

As shown in Figure 3.3 the reaction of ninhydrin with amino acids results in the separation of water and the formation of a Schiff base. The following reactions are the decarboxylation of the amino acid and in the next step an oxidative desamination. Carbon dioxide and a one C-atom shortened aldehyde are formed. This aldehyde reacts again with ninhydrin to the dye (Ruhemann's purple). The formation of the dye is proportional to the concentration of the amino acids. The method is very sensitive for amino acids, as shown in Figure 3.4.

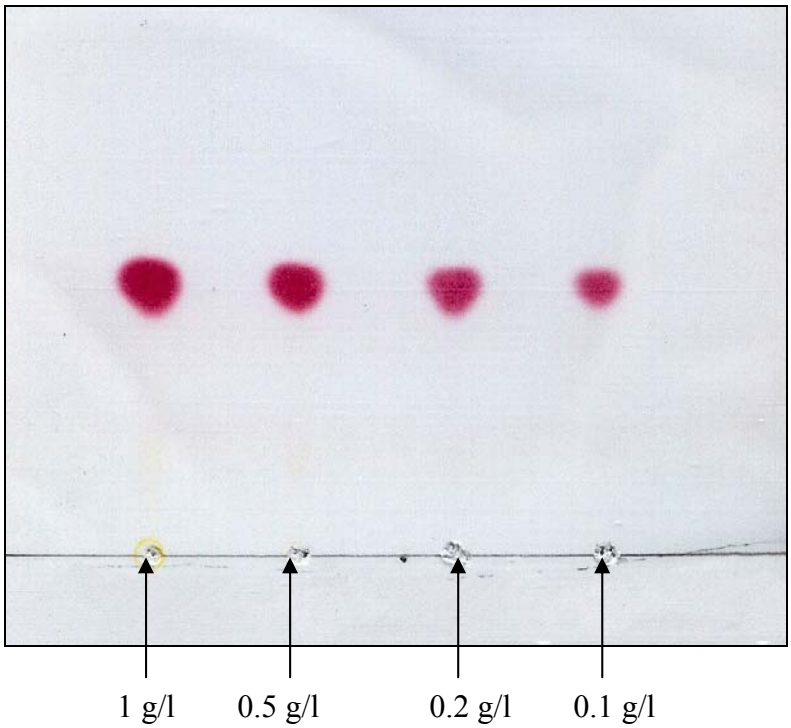


Figure 3.4: Qualitative thin layer chromatography of L-methionine samples in concentration ranges from 0.1 g/l to 1 g/l; mobile phase is n-butanol / glacial acetic acid /H_2O (12:3:5); stationary phase is immobilised on aluminium sheet: Silica gel 60 F_{254}; ninhydrin detection reaction.

3.4.5 High pressure / performance liquid chromatography (HPLC)

High pressure / performance liquid chromatography (HPLC) was used as a method to determine the concentrations of the biotransformation experiments with *Arthrobacter aurescens* DSM7330. The method was developed in the 1980s (Battilotti et al. 1988) and optimised in the 1990s (Voelkel 1993; Stehr 1996). The concept was based on the UV-absorption of the carboxyl-residues of the amino acids at 210 nm. A major problem was the absorption of other media components in this wavelength range. Evidence had shown that this was no problem for biotransformation analysis but for analysis of fermentation and cultivation samples because of the manifold composition of some complex media. An advantage of this method was the fast sample preparation. The samples were first centrifuged (15 min, 12,000 rpm; Heraeus Christ Biofuge A), then the supernatant was measured in the HPLC system. It was possible to measure the concentrations of the product L-methionine, the substrate DL-MTEH and the intermediate D-carbamoyl-methionine (see Figure 2.4). The components and the optimised conditions are shown in Table 3.8. The

calculation of the concentrations is based on calibration curves; solutions in concentration ranges between 0.1 g/l and 1 g/l were used.

Table 3.8: Components of the HPLC system and conditions for the optimised separation.

process performance	isocratic, room temperature
sample volume	20 µl
mobile phase	1% acetonitrile, 1% trifluoroacetic acid; both dissolved in H_2O, pH 2
flow rate	0.8 ml/min
column (stationary phase)	RP-18 column (Nucleosil), 250 mm length, 4mm diameter
components HPLC system	UV detector (model SPD-6AV, Shimadzu) integrator and control system (Andromeda 1.6) automated sampler (model 816. Knauer) pump (model 880 PU, Jasco)

3.4.6 Gas chromatography (GC)

The gas chromatography (GC) system (type Hewlett Packard HP5890 with automated sampler) is the most important analysis method for quantitative analysis of amino acids. The sensitivity is very high at concentration ranges above 1 mg/l. A disadvantage was the long time of sample preparation due to the derivatization process which was necessary for the measurement with this GC method. The samples were centrifuged (15 min, 12,000 rpm; Heraeus Christ Biofuge A), then the supernatant was used for the derivatization process with a special kit from the Phenomenex company (EZ:faast for free physiological amino acid analysis by GC-FID (gas chromatography flame ionization detector)). The intention of the derivatization was the transfer of amino acids from the aqueous sample to special organic solvents (in this case a mixture of octane and chloroform, the mobile phase) and the modification of the amino acids with special chemical groups in order to increase their solubilities in the organic mobile phase and to make them volatile, which was a requirement for measuring in GC systems. The derivatization kit contained an inter-

nal standard, norvaline, in a concentration of 200 mM. This is an important factor for the interpretation of results, because the concentration of other substances were determined in comparison to the known concentration of the internal standard. Details about the process are shown in Table 3.9. Another important point for the process was the temperature program for the heater (oven) to get stable base lines.

Table 3.9: Components of the GC system and conditions for the process performance.

process performance (gas flow, mobile phase)	30 ml/min N_2, 30 ml/min H_2, 300 ml/min synthetic air
analysed sample volume	1-2 µl
further adjustments	detector temperature 280°C, injector temperature 250°C
temperature program (oven)	110 – 300°C with 32°C/ min
column (stationary phase)	Chrompack CP-Sil-8CB, low bleed capillary column (15 m • 0.25 mm • 0.25 µm)
detector type	FID (flame ionization detector)
components GC system	GC system type Hewlett Packard 5890A front injector (HP7673A), automated sampler computer control system: GC ChemStation Rev.A.10.02 [1757], Agilent Technologies

The analysis kit needs to be stored at 4°C, it consists of 6 chemical solutions. The solutions and the ingredients are described in Table 3.10. In general, it is always important to mix the liquid substances for 5 – 8 seconds and to wait for at least one minute (diffusion process) before proceeding with the instruction manual (Phenomenex 2005).

Table 3.10: Reagent composition for derivatization (Phenomenex, 2005)

Reagent	Ingredients
1: internal standard solution	norvaline (0.2 mM), N-propanol 10%
2: washing solution	N-propanol
3A: eluting medium component 1	sodium hydroxide
3B: eluting medium component 2	N-propanol
4: organic solution 1	chloroform
5: organic solution 2	iso-octane
6: re-dissolution solvent	iso-octane 80%, chloroform 20%

The first step of the derivatization was the production of fresh eluting medium 3 by pooling the reagents 3A and 3B (see Table 3.10) in a ratio of 3:2. A 100 µl sample was pooled with 100 µl reagent 1 in a sample preparation vial and mixed. This step guaranteed a pH around 7 and also the internal standard norvaline was given to the sample. In the second step the solution in the sample preparation vial was passed through a sorbent tip using a syringe. The tip contained solid material which was capable of binding the amino acids. In the next step 200 µl of reagent 2 were passed through the sorbent tip (syringe). The liquid in the syringe (reagent 1 + 2) was discarded, then 200 µl of reagent 3 was given in the sample preparation vial and passed through the sorbent tip (syringe) until the liquid reached the filter plug in the sorbent tip. Then both the liquid (reagent 3) and the sorbent particles were ejected out of the tip into the sample preparation vial. The next step was the consecutive addition of reagent 4 (50 µl) and reagent 5 (100 µl) with a special pipette delivered with the kit (Drummond Dialamatic Microdispenser) to avoid cross contamination. Finally 100 µl of reagent 6 was added to the sample preparation vial. The reagents 4, 5 and 6 contained organic solvents (chloroform + iso-octane, see Table 3.10). These substances were added to extract the formed amino acid derivatives from the aqueous phase to the organic phase. The organic phase (upper phase) was transferred to an autosampler vial for the measurement with the GC system (for further and detailed information about the derivatization, see the instruction manual for the analysis kit from the Phenomenex company). An amount of 2 µl of each sample was injected and measured with the gas chromatography system.

Figure 3.5: View on the EZ:faast kit for free physiological amino acid analysis by GC-FID (Phenomenex company).

3.4.6.1 Gas chromatography - mass spectrometry (GC-MS)

The combination of gas chromatography (GC) and mass spectrometry (MS) is used to analyse mixtures. The GC serves to separate the sample components, the mass spectrometry serves to identify the constituents in context with the retention data. Principle of the MS is in general an electronic ionisation of the chemical substances and the formation of characteristical fragments which can be detected and identified on a photo plate.

The GC-MS experiments for this research were performed at the Institute for Organic Chemistry, TU Braunschweig by Dr. Till Beuerle. The used gas chromatography was an Agilent 6890 gas chromatograph, equipped with a 30 m analytical column (Phenomenex ZB5-MS, 30 m • 0,25 mm ID). The electronic ionisation (EI) mass spectrometer was a JMS-T100GC (GCAccuTOF, JEOL, Japan) model.

3.5 Bioreactor experiments

The bioreactor experiments were performed in 3 and 3.5 L scale in glass reactors (company: Infors, type: Minifors, reactor volume 5 L). A single-wall glass vessel (see Figure

3.6) with a stainless steel lid served as bioreactor. The electrodes [pH, temperature, anti-foam and pO_2 (oxygen partial pressure)], the sampling system, the stirring shaft and the connection to the stirring motor, the equipment for aeration, cooling, exhaust air and inlets for the supply of the reactor content with acids, bases, anti-foam and feeding substances over pumping systems were fixed in the lid. The automatic control technique was able to measure and to control temperature, stirring speed, pH, pO_2 (oxygen partial pressure) and foam development. The pO_2 value could be combined with the stirring speed (stirrer cascade): If the value of the oxygen parial pressure dropped under 20%, the stirring speed would automatically increase to improve the oxygen supply in the bioreactor. The stirrer was a 6 blade disk impeller type (Rushton turbine).

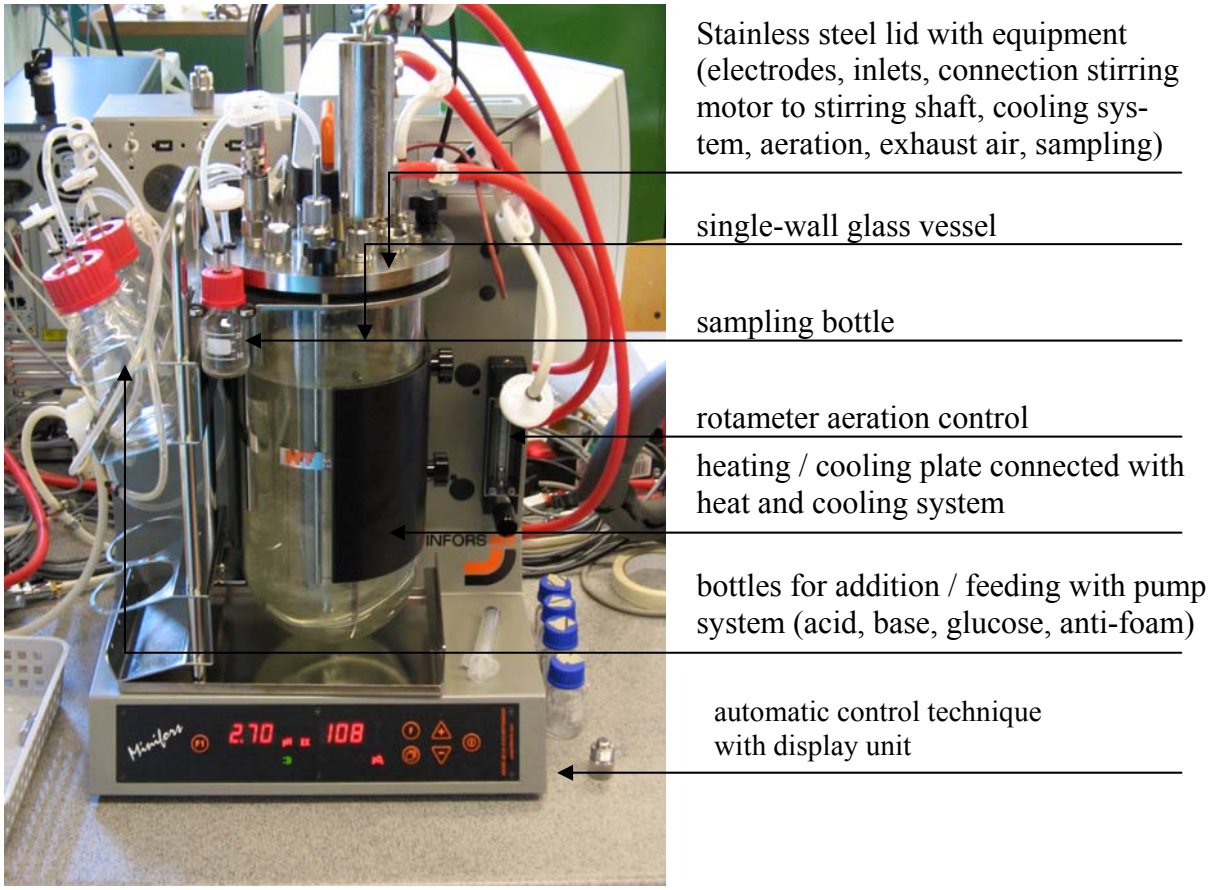


Figure 3.6: Picture of the bioreactor (company Infors, type Minifors) and the automatic control unit (controlling of temperature and cooling system, aeration rate, foam sensor, pumping systems for acids, bases, anti-foam substances and fed batch supply).

The online data acquisition was made by the computer program IRIS VIEW, the program was able to aquire the data for pO_2, pH, stirring speed, temperature and pumping rates of

feeding (acid, base, anti-foam substance). The dimensions of the bioreactor system are shown in Figure 3.7 and the following Table 3.11.

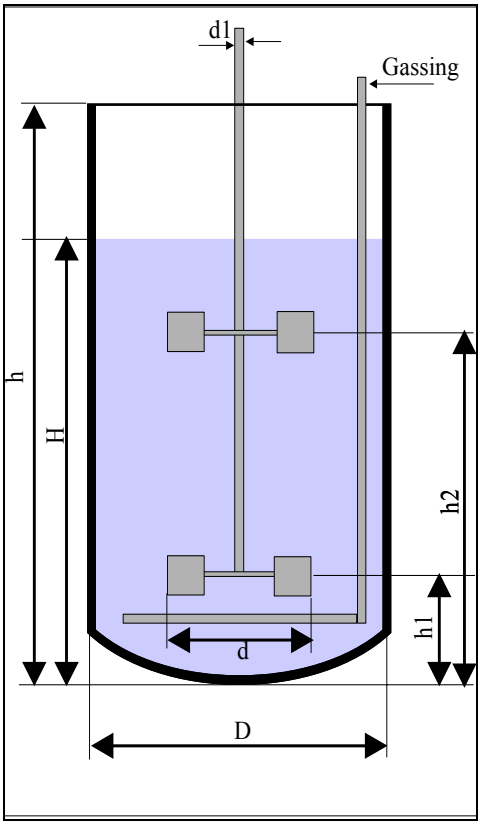


Figure 3.7 + Table 3.11: Schematic presentation of the bioreactor dimensions and register of the corresponding values.

Description	Abbreviation	Numerical value (cm) / number
Reactor fill level	H	25 cm (3.5 L)
Total reactor volume	h	33 cm (5 L)
Working volume	V	3 – 3.5 L
Reactor diameter	D	13.5 cm
Diameter of stirrer	d	6 cm
Diameter stirring shaft	d1	1.2 cm
H/D – ratio	H / D	1.85
6-Blade disk impeller	-	2
Stirrer fitting level 1	h1	8.5 cm
Stirrer fitting level 2	h2	15.5 cm
Gassing vents	-	5

In general the cultivation parameter for the fermentation with *Corynebacterium glutamicum* strains (KY10574 + DSM20300) were aquired and controlled in the following ways: temperature (30°C), pH (only aquisition), stirring speed (700 – 1,000 rpm), aeration rate: 0.5 L/(L • min), automatic increased stirrer speed by oxygen partial pressure under 20% (stirrer cascade), anti-foam monitoring and automatic addition of anti foam substance Struktol J673 (Schill + Seilacher company; dilution with water 1:10).

3.5.1 Exhaust gas analysis

The analysis of exhaust gases was measured by 2 sensors which were connected with the computer program FERMVIS. The oxygen uptake rate (OUR) and the carbon dioxide evolution rate (CER) can be counted by the method of the quasi-stationary inert gas calculation if the concentration of oxygen and CO_2 in fresh and exhaust air are known. Additionally, the aeration rate and the liquid volume in the bioreactor have to be known (Chmiel 2006). The respiratory coefficent RQ is calculated as the coefficient q_{co2} / q_{o2} (referring to molar mass).

$$Q_{O_2} = \frac{\dot{V}^N \cdot P^N \cdot X_{O_2}^E \cdot MG_{O_2}}{V_F \cdot R \cdot T^N} \cdot \left[1 - \frac{X_{O_2}^A \cdot \left(1 - X_{O_2}^E - X_{CO_2}^E\right)}{X_{O_2}^E \cdot \left(1 - X_{O_2}^A - X_{CO_2}^A\right)} \right]$$

$$Q_{CO_2} = \frac{\dot{V}^N \cdot P^N \cdot X_{CO_2}^E \cdot MG_{CO_2}}{V_F \cdot R \cdot T^N} \cdot \left[\frac{X_{CO_2}^A \cdot \left(1 - X_{O_2}^E - X_{CO_2}^E\right)}{X_{CO_2}^E \cdot \left(1 - X_{O_2}^A - X_{CO_2}^A\right)} - 1 \right]$$

Used abbreviations und indices:

V [l h^{-1}]	flow rate
V_F [l]	volume of liquid in the bioreactor
P [bar]	pressure
R = 8.314 10^{-2} bar l K^{-1} mol^{-1}	general gas constant
T [K]	temperature
MG [g mol^{-1}]	molar mass
X^E	mole fraction in fresh air (inlet)
X^A	mole fraction in exhaust air (outlet)
N	standard state (273.15 K, 1 bar)

3.6 Screening and selection system

The screening for L-methionine producing mutants was performed on the basis of *Corynebacterium glutamicum* DSM20300. The preparation of microorganisms in order to radiate them with UV-light was performed in the following steps (Gerhardt et al. 1981):

First, 50 ml SMM medium containing the strain *Corynebacterium glutamicum* DSM20300 was cultivated to a cell density of approximately $2 \bullet 10^8$ cells / ml. Then the culture was cooled on ice (10 minutes) to prevent further growth. Afterwards the culture was divided in 5 ml portions and centrifuged for 5 minutes at 6,000 rpm (Heraeus Sepatech Labofuge A) to pellet the cells. Each cell pellet was resuspended in 2.5 ml of sterile $MgSO_4$ solution (0.1 molar). The cultures were transferred to sterile Petri plates (100 mm diameter). The plates were placed on a compensator in order to move the liquid content slightly. The compensator was set up under a specially reconstructed hood (see Figure 3.8) with an UV lamp (wavelength 254 nm).

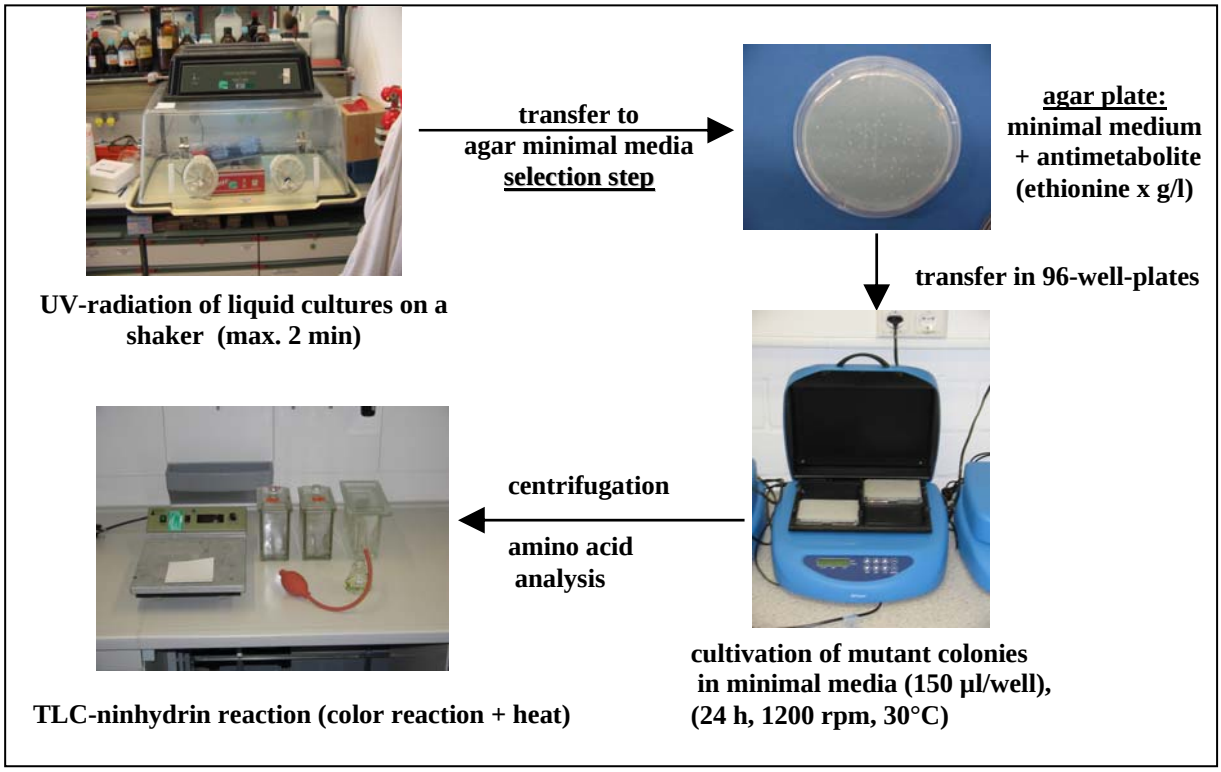


Figure 3.8: Schematical course of action for the screening experiments in order to obtain L-methionine overproducing strains (UV-radiation, selection, cultivation, analysis).

UV-radiation was optimised to a bacterial killing rate of approximately 99.9% in order to enhance the probability of producing bacterial mutant which had the metabolic modifications for L-methionine overproduction. The distance between the height of the rocker and the height of the UV-lamp were 21 cm; under these conditions the survival rate of *Corynebacterium glutamicum* DSM20300 dropped under 0.1% after 55 seconds (see Figure 3.9).

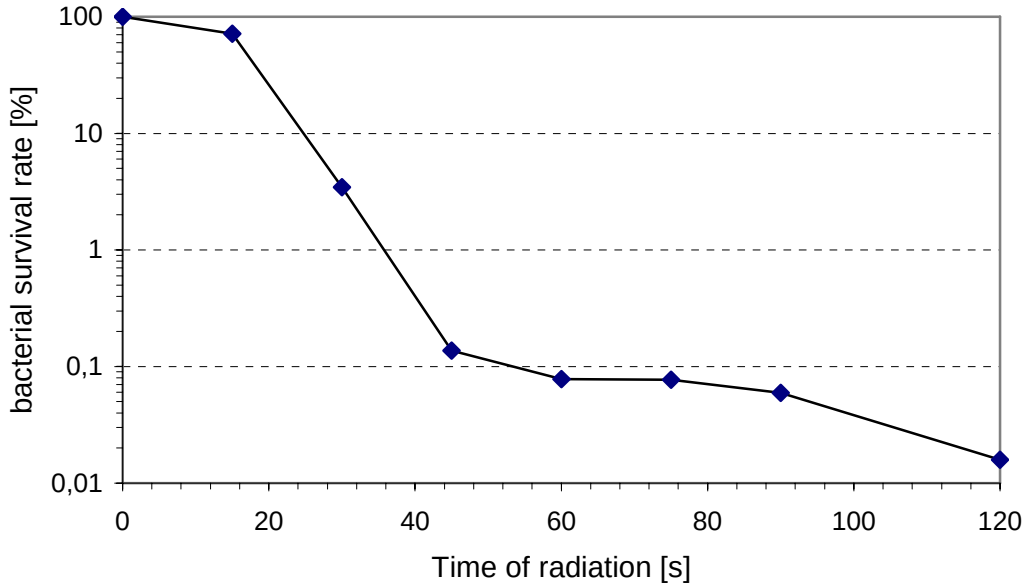


Figure 3.9: Survival rate [%] of *Corynebacterium glutamicum* by UV-radiation (254 nm) in dependence of the radiation time

After random mutation with UV-radiation the liquid was transferred to agar plates with SMM minmal medium plus different concentrations of anti-metabolite ethionine (in the most cases 2.5 g/l) in order to select a large amount of obtained bacterial mutants. The agar plates were incubated at 30°C for 48 h. It had been expected that only ethionine resistant mutants would be able to grow on these agar medias (see chapter 2.5) and, additionally, that these mutants would be capable of overproducing L-methionine. The growing colonies on the agar plates were separated by transferring the material with inoculating-loops in 96-well plates with 150 µl/well of SMM medium. The 96-well-plates were incubated on a shaker (see Figure 3.8) for 24 h by 30°C with 1200 rpm. The incubated samples were centrifuged with 6,000 rpm (Eppendorff Centrifuge 58R). In a final step, an aliquot of 5 µl was transferred under sterile conditions on thin layer chromatograpy (TLC) aluminium sheets for fast amino acid analysis. The TLC analysis was used because of the large number of samples which made it necessary to

measure in a fast and efficient way (detailed information about the thin layer chromatography (TLC) see chapter 3.4.4).

3.7 Cell disruption of *C. glutamicum* strains

The cell disruption with *Corynebacterium glutamicum* strains (KY10574 + DSM20300) was performed to measure the intracellular enrichment and concentration of L-methionine. The biomass was taken from bioreactor experiments after 48 h to get enough comparable material for several reproducible experiments.

As a first step the cultivation broth (Sorvall RC 5B plus centrifuge) was centrifuged for 20 min. with 12,000 rpm to pellet the biomass. The biomass was washed with sterile distilled water and centrifuged again under the same conditions. Then the biomass was weighed and the same amount of distilled water was added in order to get cell suspension. The cell suspension was disrupted by an ultrasonic homogenizer (Bandelin sonoplus HD2200 ultrasonic sonotrode type MS 72 and MS73), the disruption time was about 15-20 minutes. Every other minute a sample was taken for Bradford analysis to provide data of the disruption level (see chapter 3.7.1).

In a second step, the water was removed from the sample by lyophilisation. This was performed by a freeze drying system of the Omnilab company (type Alpha 1-4 LDplus) for 24 h. The obtained bio dry mass was weighed and resolved in a small amount of distilled water in order to concentrate the intracellular substances (especially the amino acids). The gained suspension was ultrafiltrated in order to cut off the higher molecular weights (MWCO). This filtration was performed by centrifugation (5,000 rpm, centrifuge Heraeus Sepatech Labofuge A) with a filtration vessel (Vivascience company, type Vivaspin 6. MWCO: 50 kDa). The cleaned solution was measured with gas chromatography (GC).

For *Corynebacterium glutamicum* DSM20300 the cell disruption was also performed using a swing mill (Retsch company, type MM2) with metal beads as grinding bodies (0.75 mm diameter). The process was carried out for 20 minutes with a grinding body filling degree of 52.5%. The cell suspension was made in the same manner as described earlier for the ultrasonic disruption.

3.7.1 Bradford assay

The protein measurement according to the Bradford method was an important step in order to determine the degree of cell disruption. During the disruption process the proteins were released from the cytosol and could be detected by this analysis. Therefore the protein concentration increased until the cell disruption degree reached nearly 100%.

The measurements were carried out with Bradford solution (Bio-Rad protein assay solution). 1,000 µl of Bradford solution (diluted in a 1:5 ratio with distilled water) were mixed with a 20 µl sample. After 20 minutes the sample was measured photometrically (Photometer, Pharmacia Biotech company, type Ultrospec 3000) in a 1 ml cuvette at 595 nm. The calibration was performed with bovine serum albumin (BSA) solutions in concentrations ranges from 0.1 to 0.6 g/l (linear area).

3.8 Protein hydrolysis of *C. glutamicum* KY10574

The protein hydrolysis of freeze-dried biomass from *Corynebacterium glutamicum* KY10574 and the following determination of amino acids (methionine, lysine and threonine) was performed at the LUFA (Landwirtschaftliche Forschungs- und Untersuchungsanstalt) in Speyer.

3.9 Chemicals and equipment

The chemicals described in chapter 3 were all purchased from the following companies in Germany: Sigma Aldrich, Fluka, Merck, Roth. The L-methionine analogue ethionine was bought from the ABCR company. The anti-foam reagent Struktol J673 was purchased from the Schill and Seilacher company. The used equipment was directly named in the context of its application. The substrates (MTEH) and the inducer (IMH) for the biotransformation experiments were received from the Evonik (former Degussa) company.

4 Results and discussion

4.1 *Arthrobacter aurescens* DSM7330: L-methionine production with biotransformation

In order to become familiar with L-methionine production and analytics a well known biotransformation was performed (Voelkel 1993; Stehr 1996). Using *Arthrobacter aurescens* DSM7330 DL-MTEH was converted into L-methionine (see Figure 4.1). Since DL-MTEH was derived chemically this procedure is not in agreement with the side conditions of our project (new European law; see introduction chapter). The analytics were performed with HPLC.

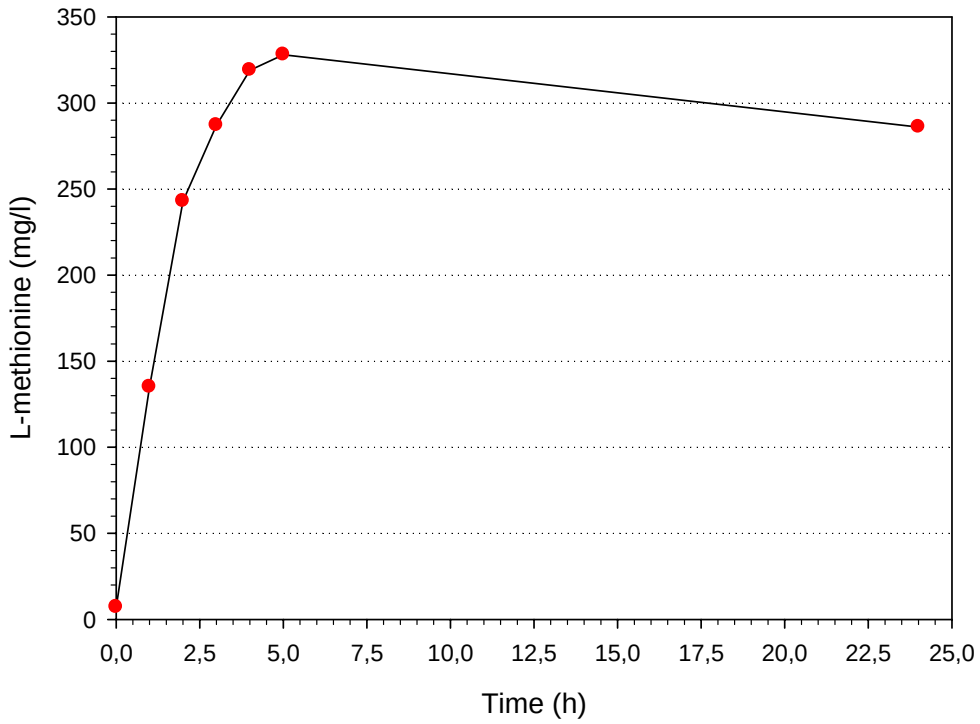


Figure 4.1: Biotransformation of the substrate DL-MTEH to the product L-methionine with whole cells of *Arthrobacter aurescens* DSM7730; biocatalysis was performed in 100 ml shake flasks (9 ml substrate solution, 1 ml of 50% cell suspension, 37°C, 24 h, 100 rpm); quantitative analysis with HPLC; for detailed information see chapters 3.2 and 3.4.5.

Figure 4.1 shows that the highest value of L-methionine production was reached after 5 h with 324 mg/l. This corresponded with a yield of 0.35 g/l L-methionine / g DL-MTEH. In former studies (Stehr 1996) it was proved that even higher yield values were possible after several optimisation experiments. After 24 h there was no further production; on the contrary, the L-

methionine concentration dropped below 300 mg/l, the balance between substrate and product became more and more inappropriate.

Although the used HPLC method was very well established for the analyzation of this biotransformation experiment, it turned to be out in the following time that this method is not suitable for quantitative amino acid measurement of biological cultivation. The reason is the manifold absorption appearance of medium components and byproducts at the measurement wavelength of 210 nm. For this reason in the following experiments with *Corynebacterium glutamicum* the gas chromatography method (see chapter 3.4.6) was developed and used.

The enantioselectivity of the biotransformation reaction is shown in Figure 4.2 with a chiral thin layer chromatography (TLC). By comparison of the references for D + L-methionine Figure 4.2 points out clearly that the enzymes from *Arthrobacter aurescens* DSM7330 cells were able to convert the L-enantiomeric substrate form to L-methionine whereas D-methionine was not produced.

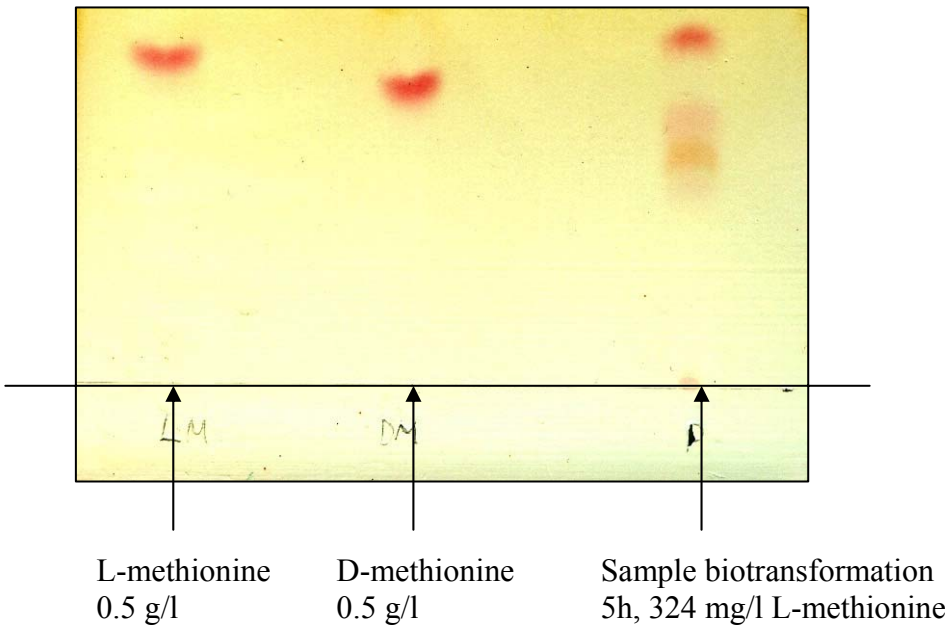


Figure 4.2: Qualitative chiral thin layer chromatography (TLC); the sample after 5 h of biotransformation (see Figure 4.1) and two reference solutions for D- and L-methionine (0.5 g/l) are illustrated, detection by ninhydrin reaction.

4.2 *Corynebacterium glutamicum* DSM20300

4.2.1 L-glutamic acid production with *C. glutamicum* DSM20300

The strain *C. glutamicum* DSM20300 is well-known as a producer of L-glutamic acid (Kinoshita et al. 1957). In this thesis the strain was important for the screening on L-methionine overproducers. Therefore it was obvious to test the quality of the strain material in relation with its most obvious characteristic feature, the L-glutamic acid production capability, by trying to reproduce former results from literature. The experiments were carried out in the shake flask scale with a complex preculture and a minimal main medium (see chapter 3.3.2 Table 3.3); the results are shown in Figure 4.3.

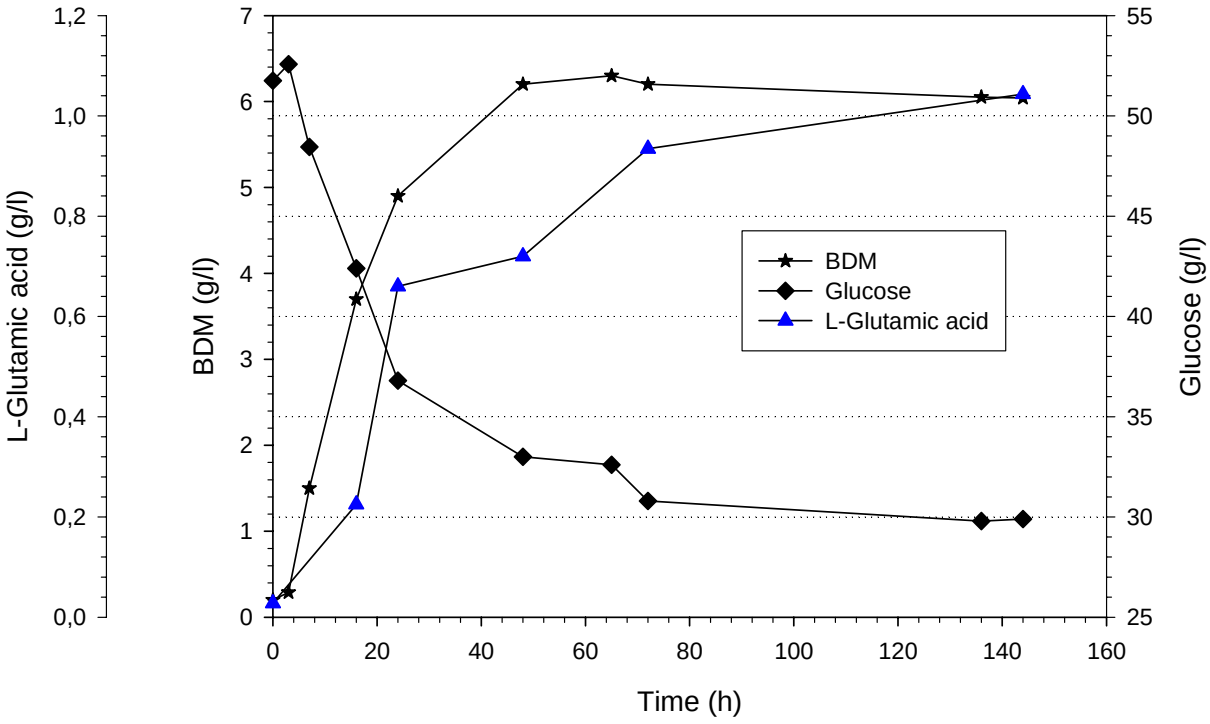


Figure 4.3: Cultivation of *Corynebacterium glutamicum* DSM20300 in the shake flask scale in L-glutamic acid production medium (500 ml shake flask, 2 baffles, 30°C, 100 rpm, 100 ml volume of MPM medium; inoculated with 2 ml from a 17 h old preculture with complex ingredients (CP)). The Figure shows the curve progressions of glucose, BDM and glutamic acid values in main medium MPM; amino acid analytics with GC.

It can be seen that the production of L-glutamic acid in the main medium increased rapidly in the first hours of cultivation time, especially around 20 hours. The production of L-glutamic acid began nearly without delay due to the use of the complex preculture (CP medium, Table

3.3). In the same manner a high formation of bio dry mass was observed in the first 20 hours. After 50 hours the stationary phase was achieved and the bio dry mass stayed constant at 6 g/l. The glucose was not consumed any more. The L-glutamic acid concentration was around 900 mg/l after 70 hours, the concentration increased slightly to 1 g/l in the following 50 hours, perhaps due to the L-glutamic acid from the intracellular milieu. Probably the contents from this milieu were released because of the deficiency of nutrients (carbon, sulphur, nitrogen and ammonia). An enrichment of intracellular L-glutamic acid in *Corynebacterium glutamicum* was observed in former investigations (Clement et al. 1986). The glucose level after 70 hours remained constant, so the nutrient deficiency has to be located in the other sources mentioned above.

In the literature (Kinoshita et al. 1961) L-glutamic acid concentrations around 3 g/l were obtained using this strain. The first basic approach to improve this experiment has to be the prevention of the nutrient deficiency after 70 hours. In further experiments higher concentrations of L-glutamic acid can be also received by optimising the cultivation parameter.

4.2.2 L-methionine production capability of *C. glutamicum* DSM20300

The wild type *Corynebacterium glutamicum* DSM20300 is not able to produce L-methionine in higher amounts than necessary for its own survival. The feedback inhibition and repression signalling cascades are fully activated (see Figure 2.10), they prevent the unefficient enrichment of metabolites which are not directly useful for the bacterial growth and survival. As preliminary investigation, before the beginning of the screening experiments, it was important to get results about the natural L-methionine production abilities.

In Figure 4.4 three representative cultivations with *Corynebacterium glutamicum* DSM20300 are illustrated. All cultivations were performed in shake flasks with the same conditions, the used modified SMM medium (see Table 3.2) was also used in the screening experiments (see next chapter). The direct metabolic precursors of L-methionine, L-homoserine and L-homocystine, were put to the SMM medium in order to support the L-methionine production and to diminish the feedback inhibition and repression phenomenons (see Figure 2.10).

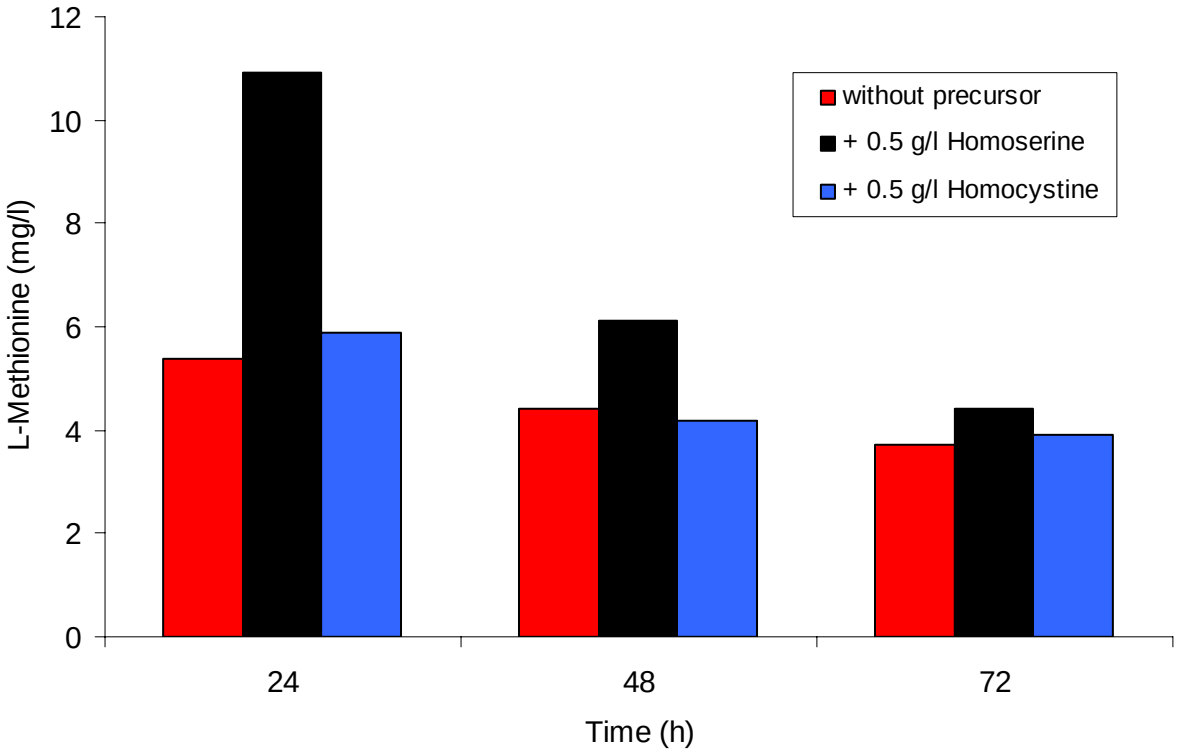


Figure 4.4: Cultivation of *Corynebacterium glutamicum* DSM20300 in the shake flask scale in SMM medium (100 ml SMM medium in 1 L shake flask (2 baffles), 30°C, 120 rpm; inoculated directly from BY agar plates; the L-methionine precursors in metabolic pathway, homoserine and homocystine were given to the SMM medium; amino acid analysis with GC.

In pure SMM medium 5.5 mg/l L-methionine was measured after 24 h, which decreased in the next 48 h below values of 4 mg/l. In the medium with 0.5 g/l homoserine 11 mg/l L-methionine were produced in the first 24 h, after that L-methionine was metabolized to 4 mg/l in the next 48 h. The results for the medium with the addition of 0.5 g/l homocystine were comparable to the results with the pure SMM medium. Therefore, it can be concluded that the addition of metabolic precursors of L-methionine does not result in a notable enhancement of L-methionine production. Only in the first 24 h a slight accumulation of L-methionine in the cultivation with the SMM medium plus homoserine was observed, but this product was consumed in the following cultivation time. To summarize, it can be pointed out that the addition of precursors was not appropriate to increase the production level of L-methionine significantly. The basis L-methionine level around 4 mg/l is necessary for the microorganism to provide his own metabolism with the essentiell amino acid L-methionine.

4.2.3 Screening for L-methionine overproducers with *C. glutamicum* DSM20300 with random mutagenesis and following selection

The main problem of the screening system was the selection process after UV-radiation. The UV-radiation was working well and quickly (see bacterial killing curve in Figure 3.9). The high amount of obtained mutants was in fact the real problem. The selection system is based on the theory that only mutants which are resistant to the L-methionine analogues (e.g. ethionine) are able to grow on media with these analogues and that these mutants have the capability of producing L-methionine.

The colony growth of *Corynebacterium glutamicum* DSM20300 is shown in Figure 4.5 in comparison to the concentration of L-ethionine. The influence of L-ethionine concentration on colony growth inhibition began at a concentration of 2 g/l. A slight reduction of the colony amount was observed in the first 24 h (85%), but the amount of colonies increased again on the following days to approximately 90%. There was a reduction of colony growth to 50% using 5 g/l of ethionine in the first 24 h, but only the high concentration of 10 g/l ethionine could prevent the colony growth in the first 24 h totally. On the second day the amount of colonies increased again over values of 50%. L-ethionine is a very expensive substance, the use of 10 g/l cannot be recommend under economic considerations for laboratory experiments. In addition, concentrations around 10 g/l L-ethionine did not only have effects on L-methionine but poisonous impacts on the whole biosynthesis. There were also attempts to work with other anti-metabolites (methyl methionine, norleucine), but the potency of these substances in growth inhibition was even lower. In the screening system L-ethionine concentrations of 2.5 g/l in SMM medium were used as selection step after UV-radiation as a compromise between economic costs and inhibition effects.

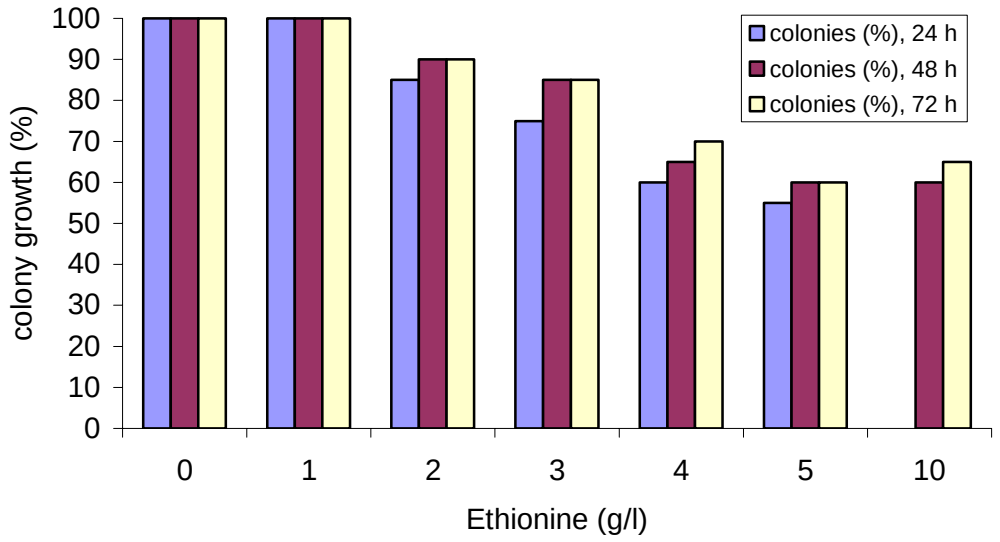


Figure 4.5: Cultivation of *Corynebacterium glutamicum* DSM20300 on agar plates in SMM agar medium with different concentrations of the L-methionine anti-metabolite / structural analogue L-ethionine; presented is the dependency of colony growth inhibition after 24 h, 48 h and 72 h with the concentration of L-ethionine.

The first experiments resulted in another problem. The screening was performed as described in chapter 3.6. The assumption that all colonies which are able to grow on L-ethionine are also L-methionine overproducers proved to be wrong. The theoretical mechanism which leads to this theory is described in chapter 2.5. The resistance of the colonies to L-ethionine is a necessary criterion, but - and this is the crucial point - not a sufficient criterion. It is therefore unevitable to measure the whole amount of colonies which are able to grow on media with distinct concentrations of L-ethionine. It was attempted to optimise the efficiency of this process and a measurement throughput of 500 – 600 samples per week was reached. But this was not enough to get a real chance to find an overproducer due to the millions of colonies received after the UV-radiation. An attempt to pick up the first appearing colonies on the agar plates for the first measurements with thin layer chromatography (TLC) assuming that they were most of all capable of producing L-methionine was not successful.

A typical screening result obtained with thin layer chromatography (TLC) is shown in Figure 4.6. The spot on the left side (L-methionine 1 g/l) and the other one on the right side (L-glutamic acid 1 g/l) of the TLC are standards, the other samples in the middle represent strain material of *C. glutamicum* DSM20300. This material was treated with UV-radiation and following selection by cultivating in medium in the presence of the anti-metabolite L-ethionine. The TLC is a fast visual analysis method. Further precise analysis with gas chromatography

might be used if the results in TLC indicated L-methionine production. The comparison of the spots in Figure 4.6 shows that there is production of L-glutamic acid, but no production of L-methionine. The wild type strain DSM20300 is able to produce L-glutamic acid, thus it is possible and probable that the derived mutants keep this characteristic feature after UV-radiation treatment.

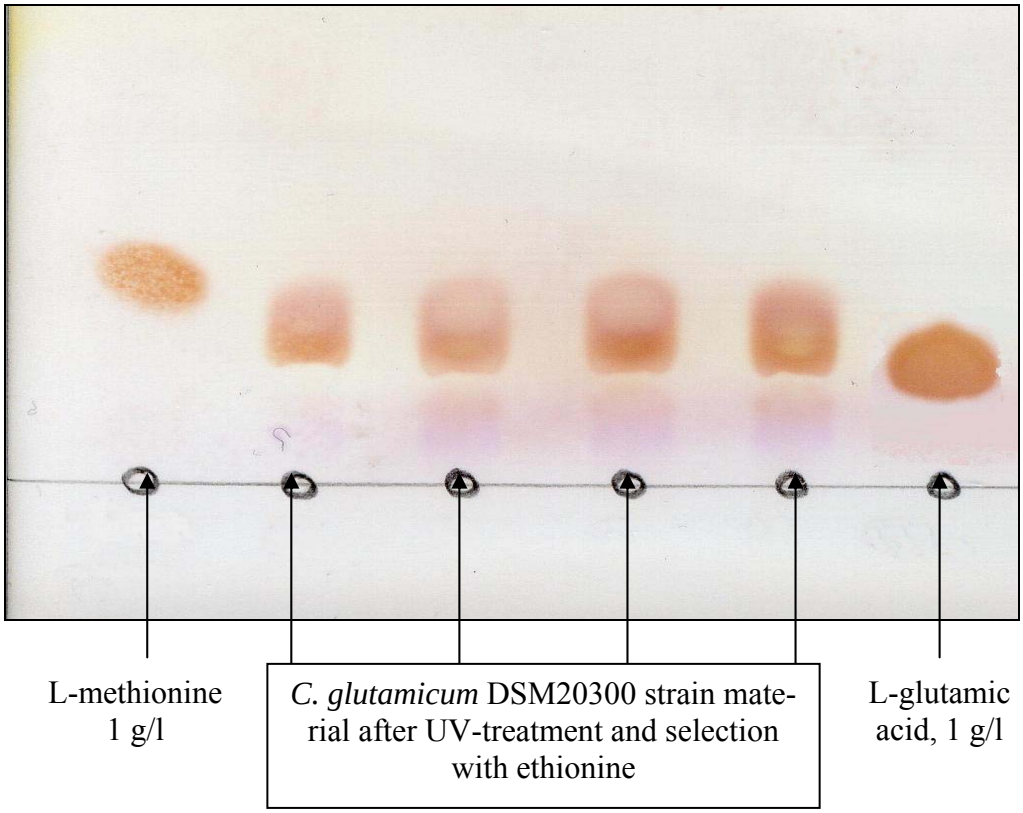


Figure 4.6: Qualitative thin layer chromatography of *Corynebacterium glutamicum* DSM20300 strain material samples after treatment with UV-radiation and anti-metabolite treatment (ethionine); standards are solutions of L-methionine and L-glutamic acid (1 g/l), detection with ninhydrin.

There might be better chances to develop a L-methionine overproducer with the strain *Corynebacterium glutamicum* KY10574. This strain is able to produce L-methionine in higher amounts (see following chapters). It could be easier to improve the properties of this strain by random mutation methods than to develop an overproducer from strain material which is not able to produce L-methionine in notable amounts. The feedback inhibition and repression signal system in *C. glutamicum* KY10574 has been already deregulated, for this reason the chances to get further success in strain treatment with random mutation increase.

4.3 *Corynebacterium glutamicum* ATCC21608

There were several experiments in the shake flask scale to observe the amino acid production of the potential L-methionine producer *Corynebacterium glutamicum* ATCC21608. In the following shake flask cultivations of this strain in F1 medium (see Table 3.6) and in KH1 / KH2 medium (see Table 3.4 / 3.5) are presented and explained. A growth of this strain in pure SMM minimal medium could not be observed, the strain demanded for more nutrients as available in basic mineral media. This is a typical side effect of random mutation procedures, the probability of additional unrequested mutations is extremely high. As a result the new strain shows in many cases growth deficiency in basic minimal media. This was not a major problem in the cultivation with the KH1 / KH2 media because the KH1 medium for the preculture had complex ingredients and the KH2 medium was inoculated in the next step with KH1 complex medium. The F1 medium is a minimal medium, but it contains many additional ingredients like mineral elements, vitamins and protocatechic acid, a chelating compound which can be very useful for enhanced growth of *Corynebacteria* cell mass (Liebl 1989).

The cultivation of the strain *Corynebacterium glutamicum* ATCC21608 in a complex preculture KH1 and following KH2 minimal medium is shown in the Figure 4.7. The minimal medium was inoculated with the preculture in a ratio of 1:11. Therefore, the curves do not start at zero in the graphs. The preculture contained particularly L-lysine in higher amounts around 300 mg/l after 24 h (no Figure), but there was no L-lysine production during the following cultivation in minimal medium anymore (see Figure 4.7).

The impact of amino acid components of the complex media on the process of production is always difficult to predict and hardly describable. It is also possible that there are unreproducible conversions of complex media components during the cultivation time which can increase the concentration of amino acids temporarily. Thus it is complicated to optimise a complex medium for microbial overproducers of amino acids due to nonexistent knowledge about the exact composition of every new media charge. Additionally, the costs for industrial process performance are much higher using complex ingredients.

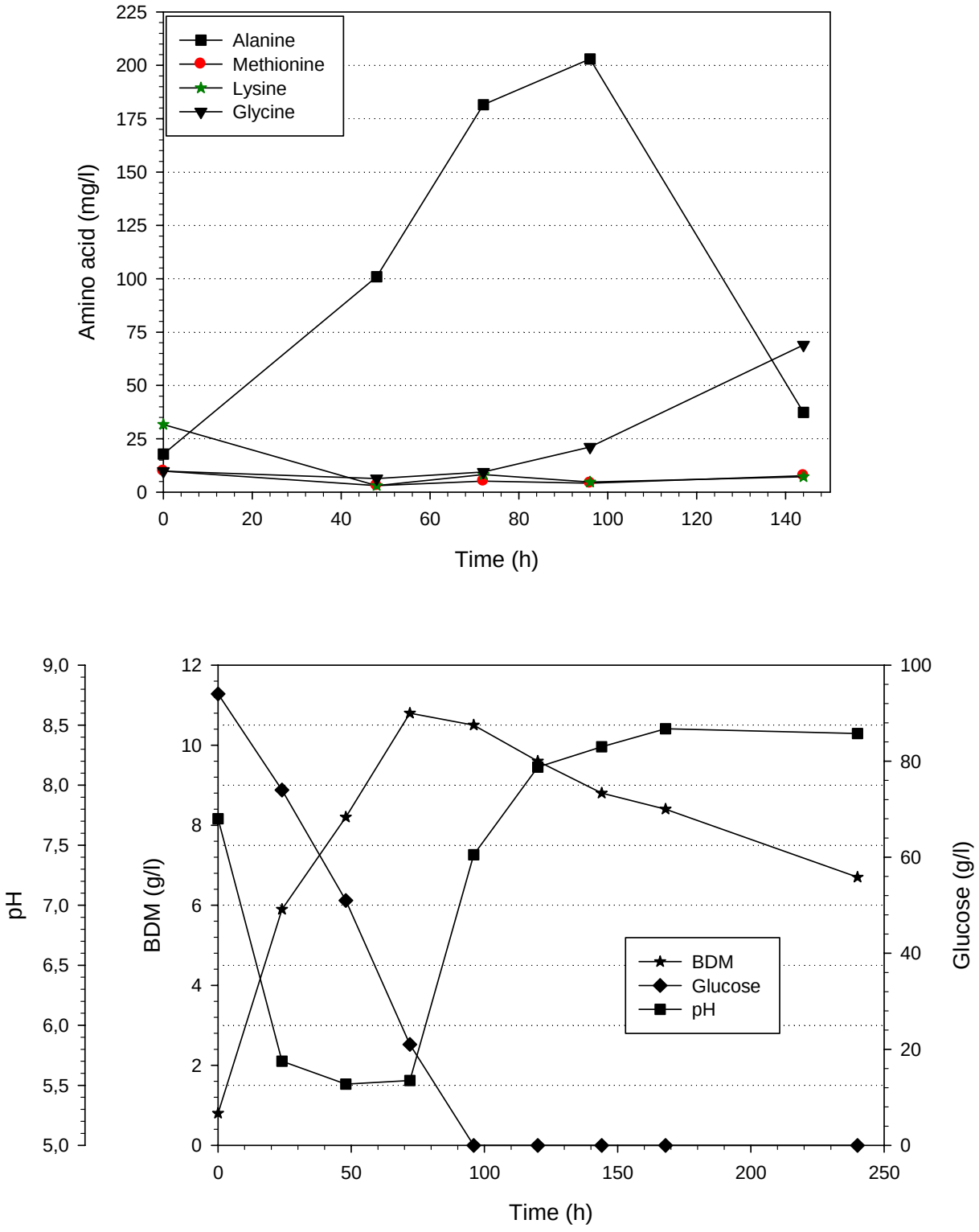


Figure 4.7: Amino acid production and development of bio dry mass (BDM), glucose and pH during the cultivation of *C. glutamicum* ATCC21608 in KH2 medium (**100 g/l glucose**) over 144 h in the shake flask scale (110 ml in 1L volume shake flask, 2 baffles, 120 rpm, 30°C); inoculated with 10 ml from complex preculture KH1 (100 ml in 1 L volume shake flask, 2 baffles, 120 rpm, 30°C, 24 h); amino acid analytics with GC.

Production of L-methionine could not be observed in levels higher than necessary for the support of the own metabolism of the strain *C.glutamicum* ATCC21608. On the other side the amino acid L-alanine was produced in concentrations around 200 mg/l after 96 h. This was also observed in another minimal medium (F1), shown in Figure 4.8.

The connection between L-alanine production and the glucose amount is quite clear to see in the Figures 4.7 and 4.8. L-alanine was produced as long as the glucose was not consumed totally. After that the L-alanine concentration began to drop quickly whereas the glycine concentration began to increase. This phenomenon also took place with another medium (F1), to be seen in Figure 4.8. But in that case there was also a high increase of L-lysine concentration at the end of the cultivation.

It could be possible that L-alanine is converted to glycine by cutting off the methyl group in order to supply the strain with carbon due to the background that glucose as the original carbon source has been consumed totally at this point in time. Under conditions of nutrient deficiency amino acids are degraded and converted in order to maintain the metabolic activities.

L-alanine and glycine are glucogenic amino acids, they are degraded through transamination to pyruvate (see Figure 2.12). Pyruvate can be converted in the next step by gluconeogenesis to glucose. A conversion from alanine to glycine is supported by these facts according to the same biochemical degradation pathway.

In contrast, L-lysine is a ketogenic amino acid (Figure 2.12), the carbon atoms are degraded to acetyl-CoA and acetoacetate. Humans and animals do not possess a metabolic pathway for the conversion of these biochemical products to glucose (Voet et al. 2002). Therefore, a conversion from L-alanine to L-lysine under glucose limited conditions is very improbable.

The pH graph in Figure 4.7 showed a characteristic course, it decreased during glucose consumption, probably according to the output of products with slightly acidic properties. Then, the pH increased rapidly in the basic area around 8.5. There are several reasons for this like the release from basic cell contents after cell collapsing and the production change to other substances under carbon source limited conditions.

The stagnation of the bio dry mass as shown in Figure 4.7 (stationary phase began at 75 h) was nearly parallel to the complete glucose consumption. The cells were not able to proliferate under carbon limiting conditions. The fast bio dry mass development during the first 96 h showed the growth ability of these microorganisms in the exponentiell growth phase using the available nutrients in an efficient way. The measurement of the BDM, however, turned out to be complicated due to the unsoluble $CaCO_3$. This led to inaccurate results in BDM, and thus the measurement of the optical density (OD) was not performed.

In further experiments it could be observed that the strain *Corynebacterium glutamicum* ATCC21608 was able to grow on F1 minimal medium which was originally designed to cultivate the strain *Corynebacterium glutamicum* KY10574 (see later). The results are shown in Figure 4.8. The strain ATCC21608 showed similar production properties in this medium compared to the cultivation in KH2 medium. L-alanine was produced in concentrations around 175 mg/l at the top after 72 h as long as the carbon source glucose was not consumed totally. Afterwards the L-alanine concentration began to drop, on the other hand the L-lysine concentration began to rise.

Additionally, a remarkable point is the cell growth velocity in the F1 medium (see Figure 4.8). In comparison to the cultivation shown in Figure 4.7 higher bio dry mass values could be reached in this experiment (13 g/l in maximum after 50 h compared to 10.5 g/l after 75 h in the former shake flask cultivation). The cultivation in F1 minimal medium outstripped the combination of complex preculture (KH1 medium) and minimal production medium (KH2 medium) in bio dry mass formation and cell growth velocity. Therefore it can be concluded that the F1 minimal medium contents are very well adapted to the nutrient demands of *C. glutamicum* ATCC21608 cells.

Higher L-alanine concentrations might be possible with more glucose in the F1 minimal medium (20 g/l in this case, Figure 4.8). But there were no higher results in L-alanine production (around 200 mg/l) using KH2 medium (Figure 4.7), which contained 100 g/l glucose. Therefore, it would be more promising to investigate other basic nutrient concentrations in the media like phosphate, sulphate or nitrogen in order to enhance the L-alanine production with *Corynebacterium glutamicum* ATCC21608.

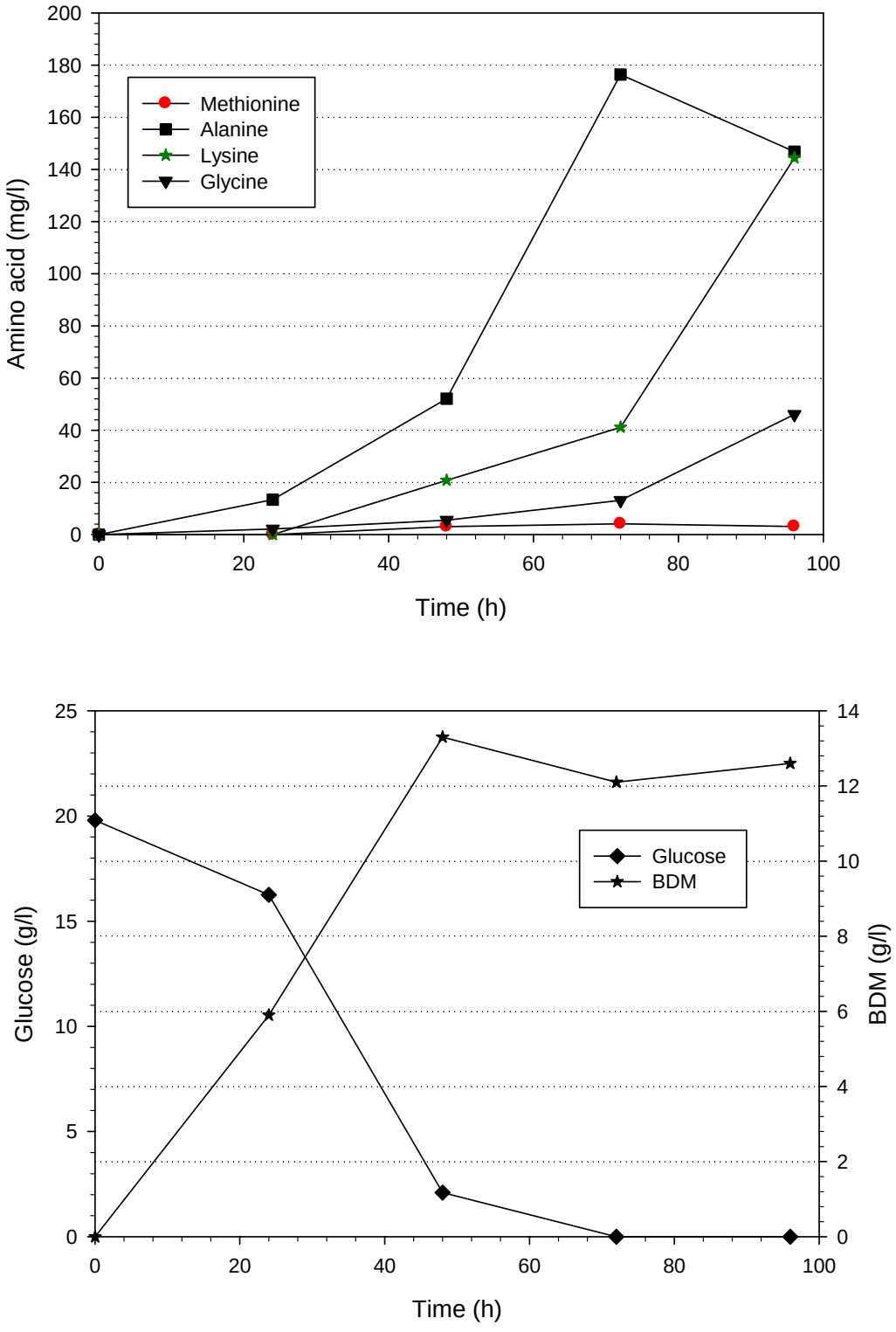


Figure 4.8: Amino acid production and development of bio dry mass (BDM) and glucose during the cultivation of *Corynebacterium glutamicum* ATCC21608 in the shake flask scale in F1 medium with **20 g/l glucose** (100 ml F1 medium in 1 L volume shake flask, 2 baffles, 30°C, 120 rpm); directly inoculated with a loopful of cells from BY agar plates; amino acid analysis with GC.

On an industrial scale L-alanine is produced from DL-aspartic acid by means of immobilized *Pseudomonas dacunhae* cells, the responsible enzyme is the L-asp-β-decarboxylase (Furui et al. 1983). Most organisms are able to produce DL-alanine in direct fermentation due to the existence of the enzyme alanine racemase. In 1993 a report was published describing the procedure to get 46 g/l D-alanine from *Bacillus lactofermentum* (Yahata et al. 1993). L-alanine can be produced in amounts of 75 g/l in a fermentation process with an *Arthrobacter oxydans* strain (Hahimoto et al. 1994).

Originally the strain ATCC21608 was bought from the ATCC (American Type Culture Collection) because of the indication of L-methionine production (Nakayama et al. 1973). Despite several attempts to reproduce these experiments to obtain L-methionine, there was no success gaining L-methionine with the material obtained from the ATCC. There are various possible reasons: e.g. the strain material was stored at the ATCC at the beginning of the 1970s, the properties of the strain material could be altered at this long storage time.

4.4 *Corynebacterium glutamicum* KY10574

4.4.1 First cultivation in the shake flask scale

The strain *Corynebacterium glutamicum* KY10574 was obtained from the Japanese company Kyowa Hakko Kogyo Ltd.. The first information about cultivation techniques and suitable media came from this company. According to this data the strain was cultivated first in a complex medium (KH1, see Table 3.4) for 24 h. After that the minimal main medium (KH2, see Table 3.5) was inoculated in a ratio of 1:11. This cultivation was carried out in two scales (1 ml preculture KH1 plus 10 ml minimal medium KH2 in a 250 ml shake flask vessel; 10 ml preculture KH1 plus 100 ml minimal medium KH2 in a 1 L shake flask vessel). The results in both scales were comparable; in the following the results for the cultivation in the bigger scale are presented.

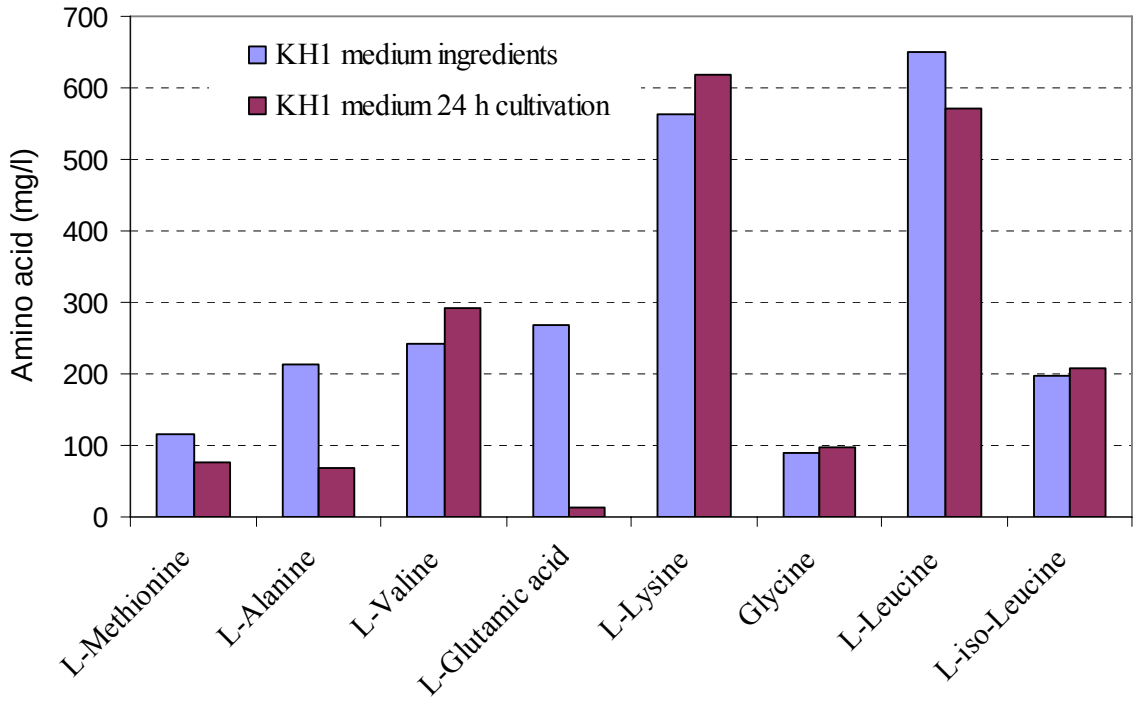


Figure 4.9: Cultivation of *Corynebacterium glutamicum* KY10574 in the shake flask scale in KH1 medium (100 ml medium in 1 L volume shake flask, 2 baffles, 30°C, 120 rpm); directly inoculated with a loopful of cells from BY agar plates. Presented are the amino acid concentrations before and after the 24 h cultivation; amino acid analysis with GC.

The precultivation in a complex medium provided increased cell growth velocity on the one hand, but on the other side there was a major problem with the composition and the ingredients. Above all the amino acids, which were present in the complex medium in relatively high concentration, shown in Figure 4.9 in many cases the concentration of the measured amino acids was in many cases higher before the cultivation was started. After 24 h the concentrations of 2 amino acids, L-lysine (approximately 70 mg/l) and L-valine (around 50 mg/l) were increased in small amounts. The other amino acids had been consumed, which could be observed most clearly in the case of L-glutamic acid. The reasons for consuming and new formation of particular L-amino acids are very hard to establish, mostly because the composition of complex media is only partially known.

In Figure 4.10 the results of four amino acids which were produced in KH2 medium (see Table 3.5) with the highest concentrations are shown. The data for the other four measured amino acids (L-leucine, L-isoleucine, L-valine, L-glutamic acid) are added in the attachment (chapter 8). The microorganism was able to produce L-lysine in amounts around 700 mg/l in maximum, followed by L-alanine with over 300 mg/l after 96 h. The production of glycine and L-methionine in significant amounts started rather late (72 h); glycine was produced in concentrations above 200 mg/l at the top; the L-methionine concentration reached concentrations of 135 mg/l at the maximum after 144 h. The glucose was consumed totally after 144 h (see Figure 4.10); all amino acid concentrations dropped down after this point in time. Exceptionally, L-alanine showed a fast increase and in the next step in almost the same manner a fast decrease before the carbon source was exhausted completely.

It can be concluded from the L-alanine production data in this and chapter 4.3 that L-alanine production was stopped as soon as the glucose level fell below certain concentration levels. As mentioned before (see Figure 2.12 and chapter 4.3) L-alanine is degraded biochemically through the glucogenic pathway which allows the recycling of degradation products in gluconeogenesis to rebuilt glucose molecules. The L-lysine concentration also decreased under glucose limitation and as a ketogenic amino acid a conversion between L-alanine and L-lysine is extremely unprobable. On the other hand glycine and L-methionine are glucogenic amino acids , their concentrations increased in the L-alanine degradation phase (see Figure 4.10). L-methionine is converted directly to succinyl-CoA in the citric acid cycle, glycine is converted glucogenically just as alanine to pyruvate. Therefore, a conversion between these two amino acids is the most probable case, the conversion to L-methionine is less probable. The decom-

position of the L-alanine molecule was very fast as described and shown in Figures 4.10, 4.8 and 4.7.

The course developments for bio dry mass (BDM) and pH, shown in Figure 4.10, are similar to those of Figure 4.7. The maximum bio dry mass value was measured after 90 h with 11 g/l. The course for the bio dry mass was directly connected to the glucose level. The pH value decreased during the production phase / exponentiell cell growth phase and increased during-the stationary phase / limitation phase and in the declining phase.

L-Lysine was produced mostly in the KH2 minimal medium (see Table 3.5). It had been already produced in the preculture (see Figure 4.9), so there were distinct start conditions for the production in the minimal medium KH2. L-Lysine was produced very fast in big amounts after inoculation of the minimal medium KH2 (see Figure 4.10). These results indicated that the production of L-lysine can be shifted to other amino acids (like L-methionine and glycine, which can be detected later in the cultivation). The problem was the usage of the complex medium for precultivation. This favoured the production of L-lysine, but the strain was not able to grow directly on the minimal medium KH2.

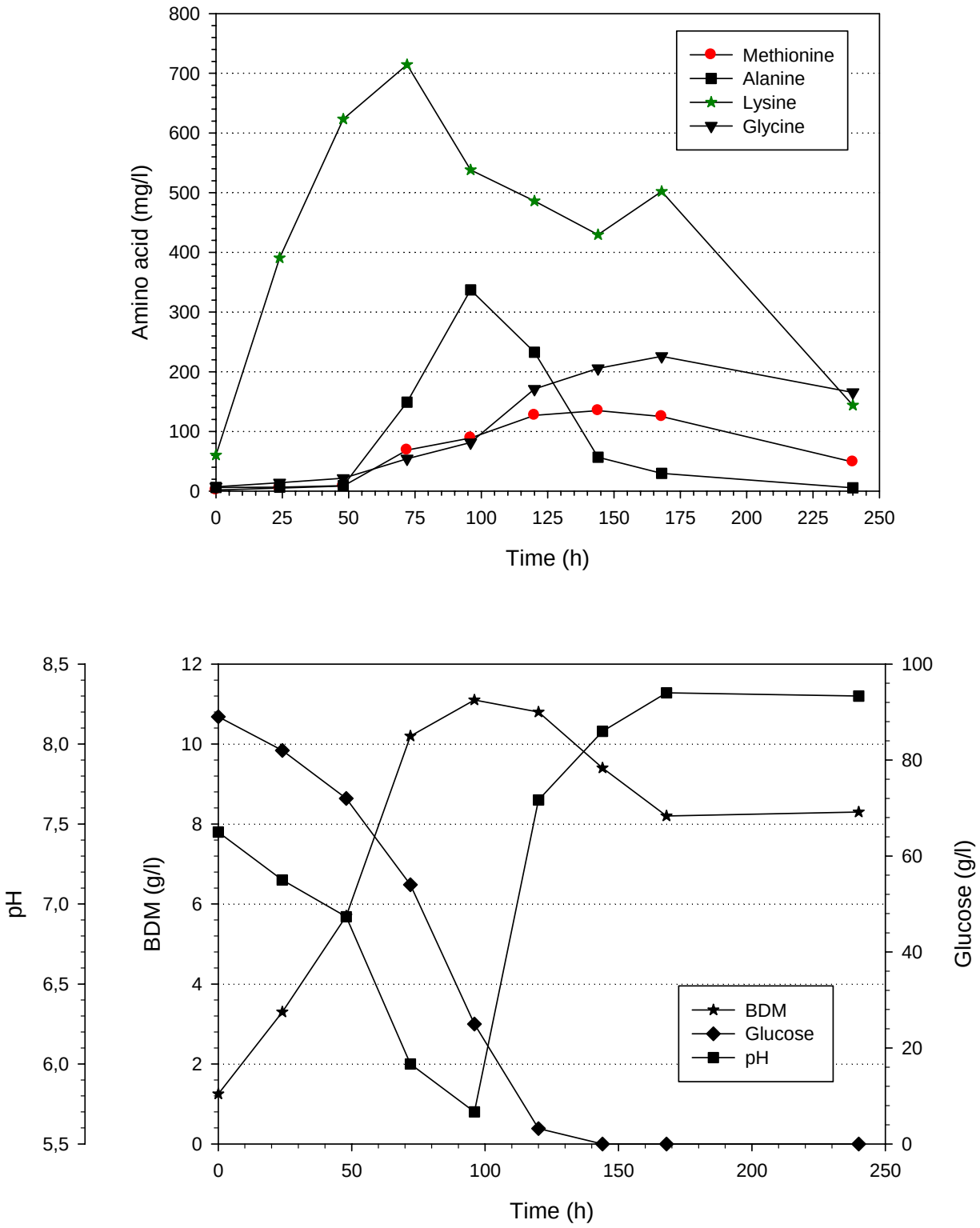


Figure 4.10: Amino acid production during the cultivation of *Corynebacterium glutamicum* KY10574 in the shake flask scale in KH2 minimal medium with **100 g/l glucose** (100 ml KH2 medium in 1 L volume shake flask, 2 baffles, 30°C, 120 rpm), inoculated with 10 ml preculture KH1 (see Figure 4.9); amino acid analysis with GC.

The production of L-lysine with *Corynebacteria* strains is well known in scientific research and industrial application (see chapter 2.3.3). It is possible to gain amounts of L-lysine with *Corynebacterium glutamicum* which are more than ten times higher than the amounts obtained with this strain (Eggeling et al. 1999). Moreover, in this work the target was the production of L-methionine in amounts as high as possible. Therefore, efforts were made to prevent the L-lysine production and to increase the L-methionine in the course of these alterations. It was attempted to change the production pattern by the alteration of significant parameters. In detail, changes were made by altering the following conditions:

- The inoculation ratio (1:11) of the KH1 (preculture) / KH2 (minimal main medium) was changed to 1:110
- The buffer system in the minimal medium ($CaCO_3$) was replaced through morpholine propane sulphonic acid (MOPS) in order to establish a more efficient and soluble buffer system
- The stirring speed was increased to higher values (120 – 210 rpm)
- Usage of centrifuged biomass for inoculation (obtained through cultivation with KH1 complex medium)

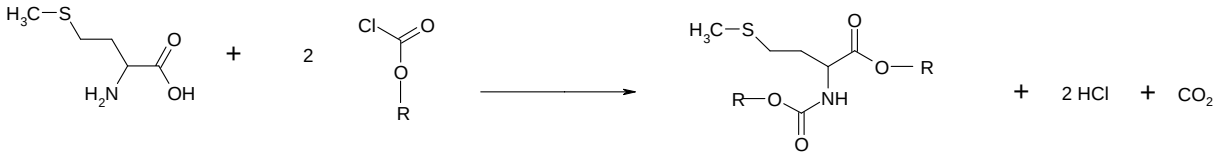
Unfortunately all efforts were unsuccessful in terms of the enhancement of L-methionine production. The only method to alter the production pattern in this case was the development of a cultivation procedure without using a complex medium. The strain *Corynebacterium glutamicum* KY10574 required just as the strain *Corynebacterium glutamicum* ATCC21608 more than the supply with basic nutrients to be able to grow. For this purpose, the minimal medium F1 was developed (see Table 3.6).

4.4.1.1 Use of GC-MS amino acid identification for KH1 / KH2 media cultivation

The cultivation as explained in the last chapter was the first successful experiment to obtain L-methionine in higher concentrations than necessary for the survival of the bacterial strain. Therefore, additional measurements of the samples after 96 h and 120 h cultivation time (see Figure 4.10) were performed at the Institute of Organic Chemistry, TU Braunschweig. It was possible to get an additional and secure proof of the accuracy of the results shown in chapter 4.4.1. The identification of the modified amino acids could be done with GC separation and

following mass spectrometry (MS) analysis which allowed a precise identification of the substances.

The first step for the identification is the exact knowledge of the derivatization process of the amino acids, performed with a GC kit for free physiological amino acids (see chapter 3.4.7). The free information from the company Phenomenex about this chemical process was not completed, as shown in Figure 4.11.



L-methionine

Figure 4.11: Chemical modification of amino acids for instance of L-methionine by derivatization due to the information of the company Phenomenex (used GC kit: EZ faast for free physiological amino acids).

In order to get additional information about the nonspecified side chains (R) and the use of catalysts, the derivatization agents 1 to 6 (see Table 3.10) were investigated by GC-MS. It could be shown that the important chemicals for the derivatization process were contents of the reagents 3A and 4. In reagent 3B the catalysator 3-methyl pyridine was identified; in reagent 4 the substance propylchloroformate was identified which indicated that the unnamed side chains in Figure 4.11 stand for propyl groups. The original measurement data of the reagents 4 and 3B are added to the attachment (chapter 8). The completed chemical reaction for the derivatization of amino acids is shown in Figure 4.12. Several scientific reports have been published about amino acid analysis and derivatization before measurement. The chemical reaction mechanism shown in Figure 4.12 is also described in principle from Husek (Husek et al. 1990). With the information about the amino acid modification it was possible to identify the fragments received from the mass spectrometry.

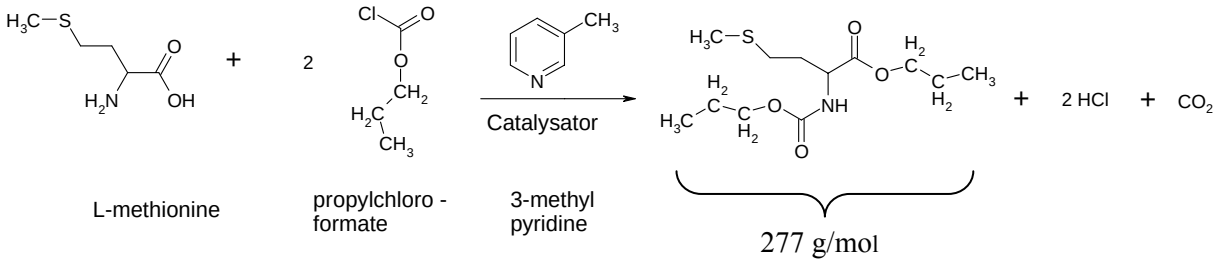


Figure 4.12: Completed chemical reaction for the modification of amino acids by derivatization before GC measurement for the instance of L-methionine after determination with GC-MS

In the next step the sample of the cultivation of *Corynebacterium glutamicum* KY10574 after 120 h (see Figure 4.10) was measured with GC-MS at the Institute of Organic Chemistry, TU Braunschweig. The sample was modified through derivatization as shown in Figure 4.12 and in the same way as all samples obtained from the cultivations. The results obtained from the GC measurement at the Institute of Organic Chemistry are shown in Figure 4.13. In addition Figure 4.14 shows the chromatographic patterns obtained with the gas chromatography at the Institute of Biochemistry and Biotechnology in order to be able to compare the results (see next page for Figures 4.13 + 4.14).

The chromatographic patterns in Figures 4.13 and 4.14 are similar, but the retention times are different. This can be referred to the different length of the used GC columns, but the material of the columns (solid stationary phase) were identical. For this reason the interactions between the samples and the GC column material were similar. The chromatogram in Figure 4.14 shows more background peaks at the base line than in the comparable chromatogram in Figure 4.13 at which a very clear baseline can be recognised.

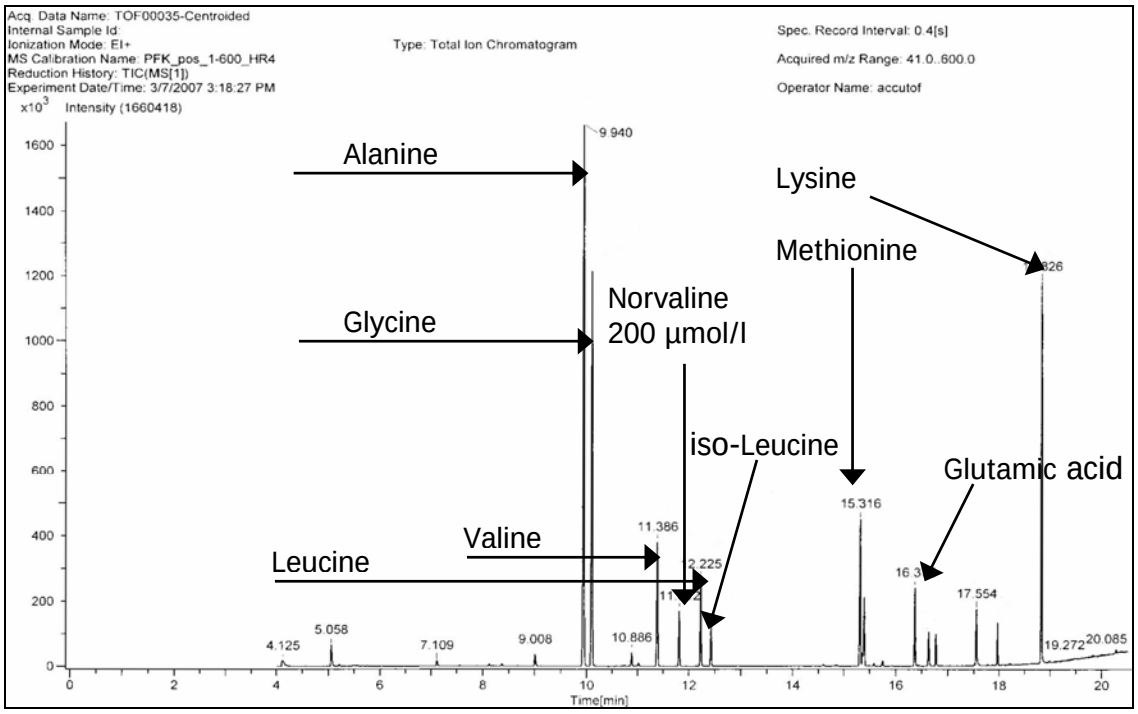


Figure 4.13: Gas chromatography (GC) results after derivatization of the cultivation sample after 120 h in the shake flask scale (see Figure 4.10); this experiment was performed at the Institute of Organic Chemistry, TU Braunschweig. Gas chromatograph type Agilent 6890, equipped with a 30 m analytical column (Phenomenex ZB5-MS, 30 m • 0,25 mm ID).

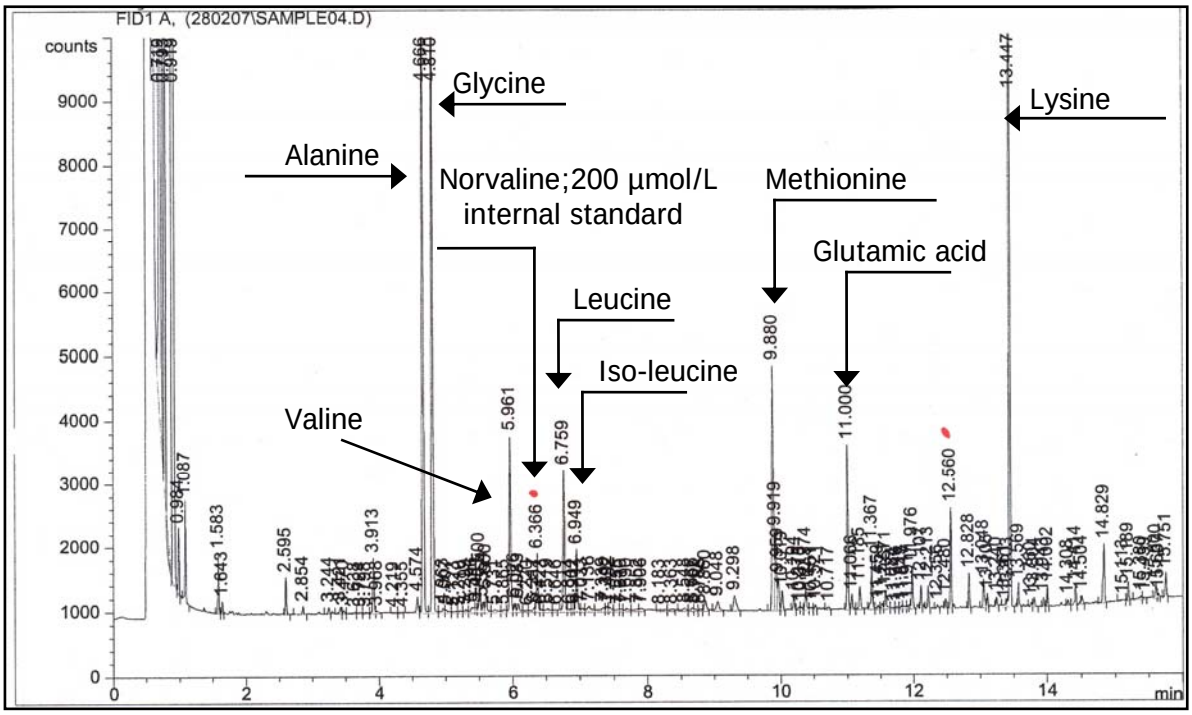


Figure 4.14: GC results after derivatization of the cultivation sample after 120 h in the shake flask scale (see Figure 4.10); this experiment was performed at the Institute of Biochemistry and Biotechnology, TU Braunschweig. Gas chromatograph type Hewlett Packard HP5890, equipped with column: Chrompack CP-Sil-8CB, low bleed capillary (15 m • 0.25 mm • 0.25 µm).

In Figures 4.13 + 4.14 some chromatographic peaks cannot be allocated precisely to a distinct amino acid or, in general, a definite chemical compound. These unidentified compounds have to hold an amino group in their chemical structure. This is referred to the derivatization process before measurement which filtered out all substances without those properties. On the other side this method allowed to identify 8 amino acids and the internal standard L-norvaline without any doubt, among them the most important metabolites (above all L-methionine, L-lysine, L-glutamic acid, glycine and L-alanine) which can be produced with *Corynebacterium glutamicum* bacteria. The fact, that the strain *Corynebacterium glutamicum* KY10574 was able to produce such a broad amino acid spectrum in different media proved the amino acid production variety of this strain under the described conditions.

The metabolism of L-methionine, L-lysine, L-alanine and glycine was deregulated, otherwise it could not be explained why the microorganism was able to produce these amino acids in higher values than necessary for its own supply with nutrients. Under these considerations the composition of the medium becomes more and more important, because the microorganism seems to be able to choose the metabolic production pathway depending on the offered nutrients from the available medium.

The deregulation of several amino acid biochemical pathways occurred by randomised mutagenesis. This microbiological method can improve the abilities of microorganisms to overproduce metabolites considerably (see chapter 2.5) but generates also, apart from inserting the favoured mutation (e.g. the derugulation of L-methionine feedback repression and inhibition), simultaneous inserts which lead to unrequested additional mutations with unwanted side effects.

The Figure 4.15 shows the identification of the amino acid L-methionine with mass spectrometry (MS). The amino acids were separated due to the GC method (see Figure 4.13 + 4.14), so it was possible to analyze them in a MS system. The amino acids, in this case L-methionine, were degraded by electronic ionisation. The obtained fragments from a methionine reference and a real sample are presented in Figure 4.15. The Figure presents a time of flight (TOF) graph, the fragments were detected in distinct points in time.

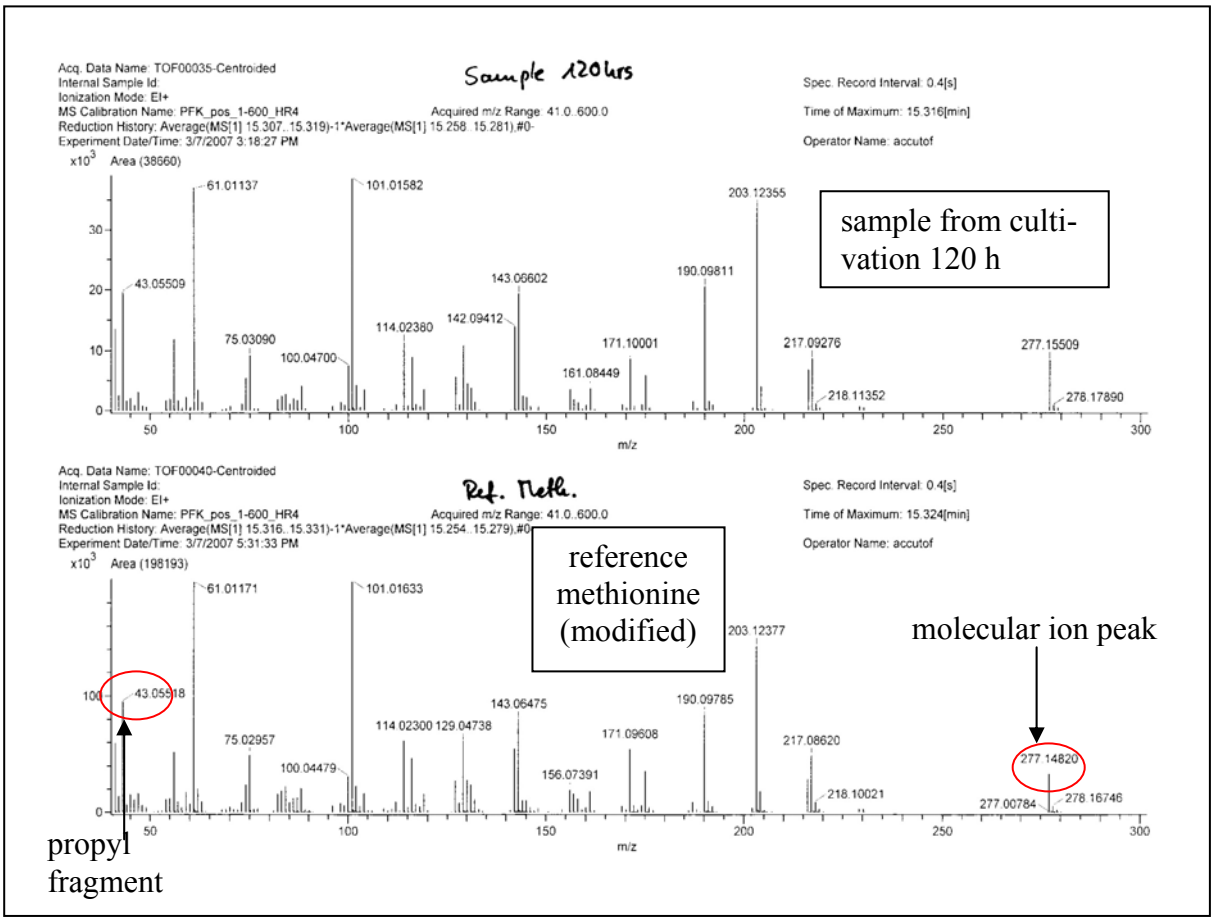


Figure 4.15: Mass spectrometry (MS) results for the example of **modified L-methionine (see Figure 4.15)** from a reference sample (at the bottom) and a cultivation sample after 120 h (at the top); see for the cultivation Figure 4.10. This experiment was performed at the Institute of Organic Chemistry, TU Braunschweig with a time of flight mass spectrometer type JMS-T100GC (GCAccuTOF, JEOL, Japan).

Each chemical compound shows significant fragment patterns after mass spectrometry (MS) analysis including molecular ions and ions from certain degradation steps. The modified form of L-methionine has a molar mass of 277 g/mol (see Figure 4.15). In the MS-TOF graph of both samples the residual unfragmented molecule can be detected (277 g/mol). Another significant fragment is the first one in both diagrams in Figure 4.15, the propyl fragment. As shown in Figure 4.12, 2 propyl groups are connected to L-methionine in the derivatization process; so the existence of these groups is another important information for the interpretation of the diagram. Nowadays, large databases exist to identify chemical substance pattern gained from MS. The mass spectrometry diagrams for the other identified amino acids (L-alanine, L-valine, L-glutamic acid, L-lysine, L-leucine, L-isoleucine, glycine) are added to the attachment (chapter 8).

4.4.2 Shake flask cultivation of *Corynebacterium glutamicum* KY10574 in F1 minimal medium

After first attempts to cultivate the strain *Corynebacterium glutamicum* KY10574 in a combination of complex medium (KH1, see Table 3.4) as preculture and a minimal medium (KH2, see Table 3.5) for production of the amino acid L-methionine, it became evident, that it was impossible to enhance the L-methionine production this way. Furthermore, the production of methionine began very late in the main culture (KH2 medium), after approximately 80 – 90 h, not including the 24 h of precultivation. Another problem was the carbonate buffer system ($CaCO_3$), which was not very efficient. It complicated the measurement of the BDM and the optical density (OD).

The strain *Corynebacterium glutamicum* KY10574 had problems growing on pure minimal media; it was unable to grow on minimal medium SMM (Table 3.2) and KH2 (Table 3.5). Therefore, the developed minimal medium F1 (see Table 3.6) was tested. It contained a broad variety of mineral elements and vitamins. The first cultivation of *C. glutamicum* KY10574 in F1 minimal medium is shown in Figure 4.16.

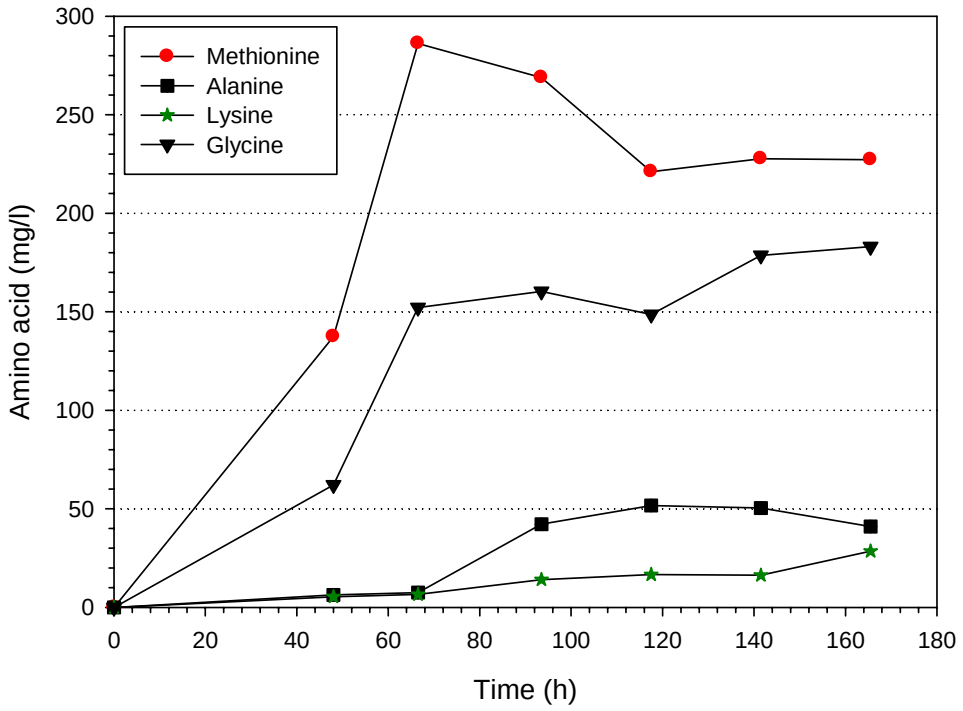


Figure 4.16: Amino acid production during cultivation of *C. glutamicum* KY10574 in F1 minimal medium, **20 g/l glucose**, 30°C, 150 rpm, 25 ml medium in a 250 ml shake flask vessel (3 baffles); inoculated directly from BY agar plates; amino acid analysis with GC.

The change of the amino acid product spectrum compared to former cultivations (Figure 4.10) is obvious. There was only a small amount of L-lysine, which was the dominant amino acid in the cultivations with KH1 + KH2 media. The amounts of glycine are comparable to those shown in Figure 4.10. However, it lasted much longer in the former cultivations with complex precultures until the production achieved the same concentrations as in this cultivation. The production of L-methionine began to start very rapidly and stopped after 66.5 h at with 286 mg/l. The concentration and the production of L-methionine were more than doubled compared to Figure 4.10. The slight decrease of concentration at the end of the shake flask cultivation can be explained by glucose limitation. In general, the microorganisms are stopping the metabolite production or they alter the production to other and easier constructed molecules. In this case (Figure 4.16) a slight product shift from L-methionine to L-lysine after the complete consumption of the carbon source could be observed. L-alanine was produced very late and only in small amounts. In the following chapter 4.4.3 it is pointed out that the L-alanine production started to increase with the presence of higher glucose levels in the medium.

Another important experience from this cultivation was the influence of the shaking rate. In the first attempts with a shaking rate of 120 rpm, the strain began either to grow only after a long adaptation phase or there was actually no cell growth at all. This is remarkable because the cultivations were performed at the beginning only in small scales with 25 ml minimal medium F1 in a 250 ml shake flask scale. The inoculation was done with a loopfull of cells from BY agar plates. With higher shaking rates (150 rpm) the time of the lag-phase was shortened for the benefit of the exponentiell growth phase. The strain *C. glutamicum* KY10574 and in general all *C. glutamicum* strains exhibited a high demand for oxygen (Gomes et al. 2005; Sharma et al. 2001). According to this data it was important to provide good aeration conditions by intensive mixing through the shaking rate and through the baffles in the shake flask walls to rip open the surface of the cultivation broth. By this procedure there was an enlargement of the liquid surface which allowed a much higher oygen uptake rate (OUR) inside the liquid medium. Especially at the beginning good aeration was absolutely crucial, since the modality of cell growth depended at first on optimised mixing conditions and oxygen supply. A real optimisation of the aeration could be better performed in bioreactors with controlled gassing in combination with an effective stirrer speed for mixing which was much more efficient than the shaking rates in the shake flask scale. This is explained in detail in the bioreactor cultivations with the strain *Corynebacterium glutamicum* KY10574 in chapter 4.4.7.

A sample, taken from the foregoing cultivation from Figure 4.17 after 66.5 h (maximum L-methionine production) was used as inoculum for the shake flask cultivation shown in Figure 4.17. The target was the enhancement of L-methionine production by transferring a preculture with high methionine concentration in a fresh medium. Due to this preculture the amino acid concentrations increased practically without a noticeable adaptation (Figure 4.17). The L-methionine maximum was reached very early after 27 h. After about 50 h most of the amino acid concentrations began to decrease. The L-methionine concentration was only slightly enhanced by 50 mg/l from 286 mg/l after 66.5 h (Figure 4.16) to 333 mg/l and 339 mg/l after 27 h respectively 51 h (Figure 4.17). The concentration of the other amino acids remained unchanged. So it was not very effective to increase the L-methionine yield using samples as inoculum like it was done in this case. The level of glucose and the glucose limitation at a distinct point in the cultivation process seemed to be the more crucial part in order to rise the amino acid formation and production.

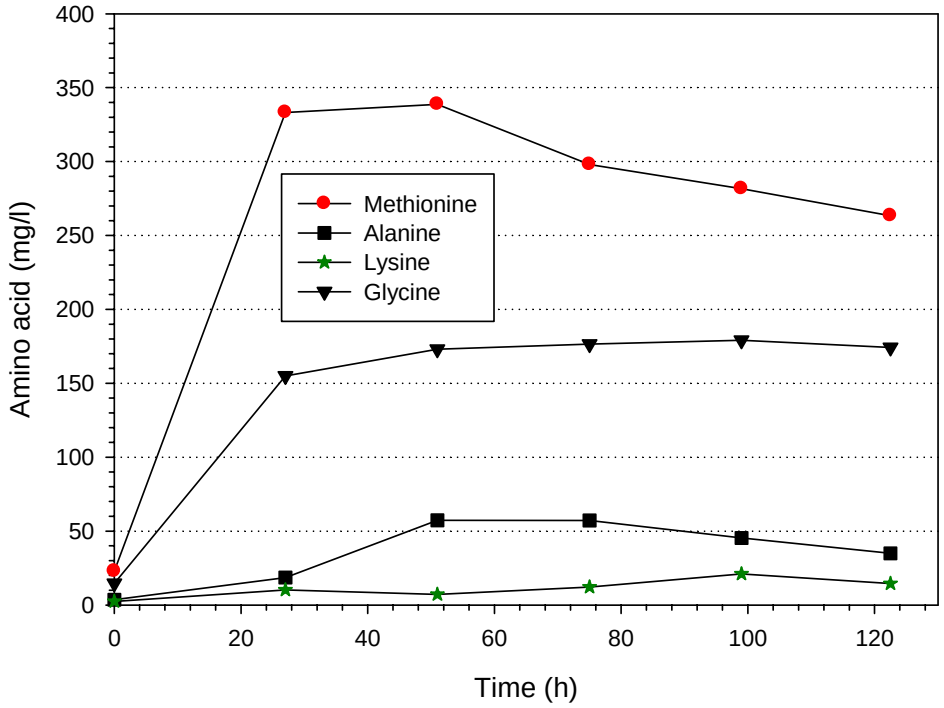


Figure 4.17: Amino acid production during cultivation of *C. glutamicum* KY10574 in F1 minimal medium, **20 g/l glucose**, 30°C, 150 rpm, 25 ml medium in 250 ml shake flask vessel (3 baffles); inoculated with preculture: 2.5 ml sample taken from the cultivation shown in Figure 4.16 after 66.5 h; amino acid analysis with GC.

To be sure that the GC-detection of methionine is in qualitative agreement with well-known colour-based methods, routinely thin layer chromatography (TLC) was used. The thin layer chromatography of 2 samples obtained from the shake flask cultivation, shown in Figure 4.17 (27 h and 51 h), reveals the results from the gas chromatography in a visual way. The retention times of the methionine spots in both samples was completely identical with the that of the methionine reference which served as an additional proof for the correctness of the measurements. In comparison to the reference it was also evident that an analysis of amino acid concentration by means of this method can be helpful to get index values, but not for quantitative determinations of the amino acid concentrations.

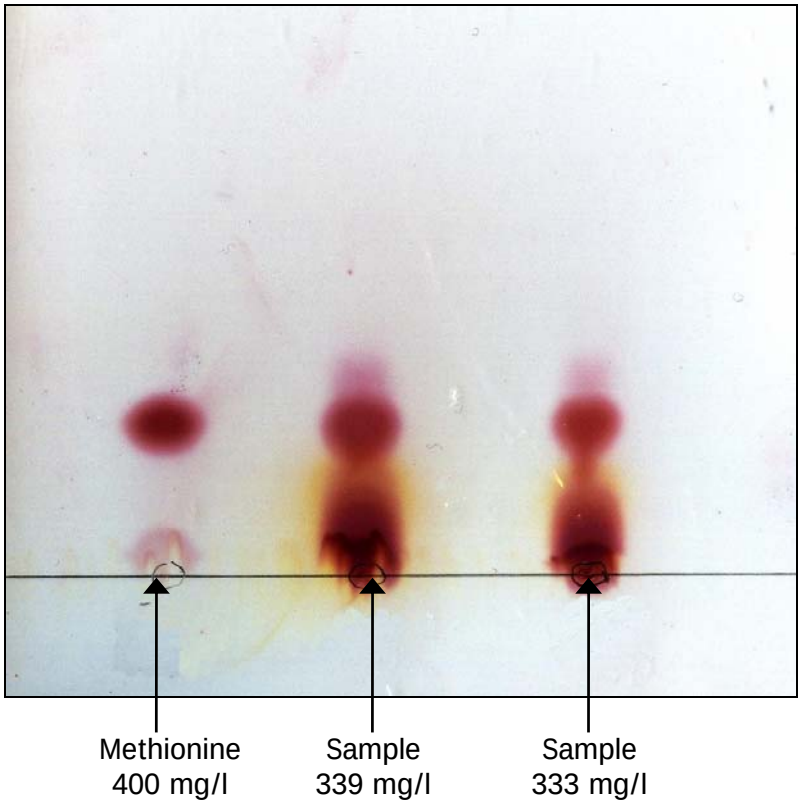


Figure 4.18: Qualitative thin layer chromatography (TLC) of the samples after 27 h and 51 h from the cultivation shown in Figure 4.17 and a reference of L-methionine (400 mg/l); detection with ninhydrin.

4.4.3 Shake flask cultivations in F1 minimal medium with different glucose levels at rising shaking rates

After first positive results obtained with the minimal medium F1 in the shake flask scale systematic investigations were done to improve the L-methionine production conditions. The first experimental series were performed to optimise the glucose level of the F1 medium and the shaking rate. The glucose level varied between 20 and 100 g/l, the shaking rates between 150 and 210 rpm. It was the target to adjust the highest possible shaking rate to enhance the oxygen transport and supply in the shake flask cultures, which seems to be very important for both a cell growth and metabolite production of *Corynebacterium glutamicum* (Sharma et al. 2001). Due to the increasing density of the cultivation liquid in the course of the cultivation time (caused by the bio dry mass development) the shaking rates were boosted gradually in the following steps: 150 rpm (0 – 45 h), 180 rpm (ca. 45 – 72 h), 210 rpm (≥ 72 h). These alterations were carried out for both scales: 25 ml medium in 250 ml shake flask and 100 ml medium in 1 L shake flask. In the following chapters there is a complete exemplary presentation of the results for the small scale (Figures 4.19 – 4.23), an example for the big scale cultivation (Figure 4.24) and a comparison of the measurements from both scales (Figure 4.25). The complete series is shown to give clear information about the direct context between the amino acid production on the one hand and the cell growth development as well as the glucose consumption on the other hand.

4.4.3.1 25 ml cultivation (250 ml shake flask) with 20 g/l glucose

The experimental series was started with 20 g/l of glucose. The L-methionine and glycine production values shown in Figure 4.19 can be directly compared with the first cultivations with this medium (F1) as presented in Figure 4.16. The variations in the product values for L-methionine and also for glycine were in comparison of both diagrams in normal ranges. L-methionine concentrations of approximately 230 mg/l after 44 h can be achieved with a glucose level of 20 g/l, glycine amounts were around 200 mg/l. Other amino acids were produced only in small amounts (< 50 mg/l) as shown for L-alanine and L-valine in Figure 4.19.

The decrease of the L-methionine concentration after 50h occurred probably due to the fast consumption of glucose (consumed after 30 h). As described before amino acids like alanine, methionine and glycine are degraded to products of the citric acid cycle leading at the end to gluconeogenesis (see Figure 2.12 and chapter 4.3). Therefore, it can be assumed that these amino acids are degraded in order to supply new glucose molecules for maintaining the microbial metabolism. These results suggest further investigations with higher levels of glucose.

The biomass reached 7 g/l after 30 h, further cell growth was not possible due to the consumed carbon source (glucose). As expected the development of the course for the optical density was parallel with the bio dry mass.

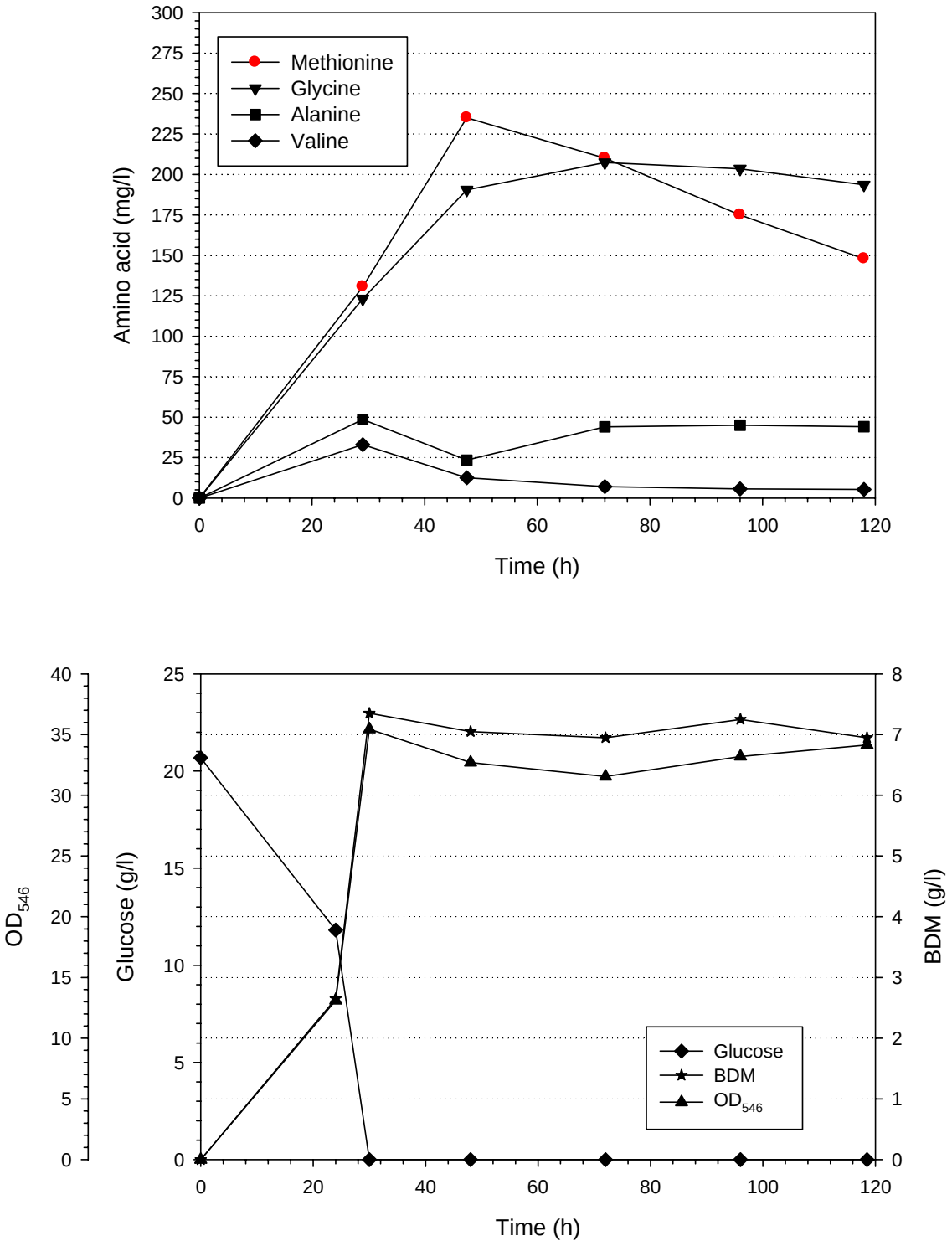


Figure 4.19: Amino acid production and development of bio dry mass (BDM), optical density and glucose (g/l) during cultivation of *C. glutamicum* KY10574 in F1 minimal medium, **20 g/l glucose**, 30°C, 25 ml medium in a 250 ml shake flask vessel (3 baffles); inoculated directly from BY agar plates; shaking rate: 150 rpm (0 – 45 h), 180 rpm (ca. 45 – 72 h), 210 rpm (≥ 72 h); amino acid analysis with GC.

4.4.3.2 25 ml cultivation (250 ml shake flask) with 40 g/l glucose

The cultivation results with 40 g/l of glucose in the F1 minimal medium are shown in Figure 4.20. The L-methionine concentration reached levels above 325 mg/l after 45 h before the amounts dropped quickly. In certain cases higher L-methionine production could be observed, but in most experiments with this glucose concentration in the medium there were concentrations around 300 – 400 mg/l L-methionine in the cultivation broth. But the exceptions with higher L-methionine amounts in the shake flasks proved a capability for advanced production.

The glucose was again consumed very rapidly due to attempts to provide high oxygen supply into the medium. The bio dry mass values were doubled compared to the experiment shown in Figure 4.19, but this fact did not yet successfully increase the L-methionine production significantly. The production of glycine is increased slightly from 200 mg/l (Figure 4.19) to values of 250 mg/l in Figure 4.20. The L-valine concentrations are on low levels, only after 30 h a value above 50 mg/l could be measured.

The L-alanine concentration rose quickly after cultivation had started but dropped in the same way immediately after the consumption of the carbon source. The instantaneous decrease of L-alanine concentrations under glucose limited conditions was characteristical for this amino acid, no other amino acid reacted in this way with the glucose level. This is most probably due to biochemical degradation in order to gain new glucose molecules through gluconeogenesis (Figure 2.12, chapter 4.3, chapter 4.4.3.1).

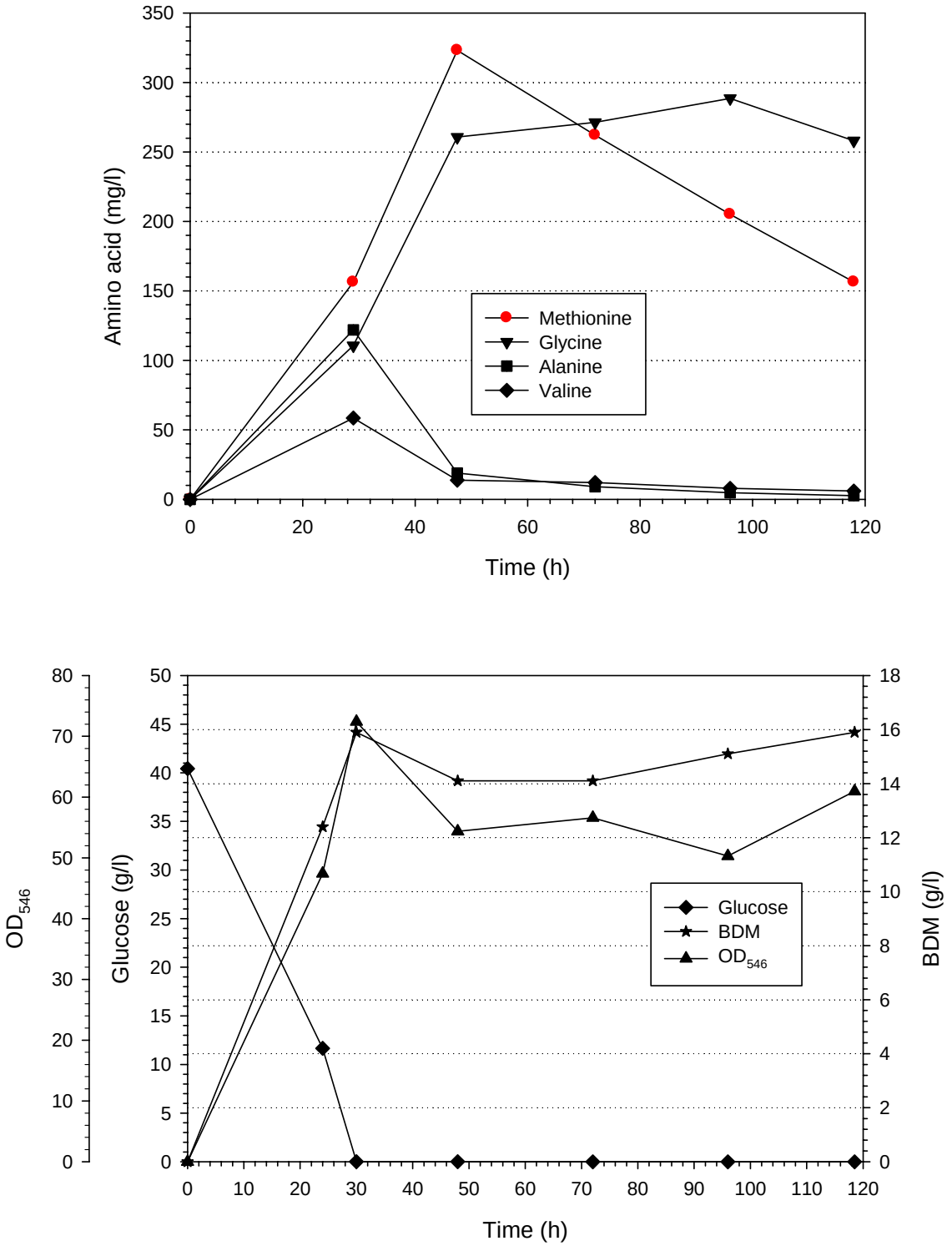


Figure 4.20: Amino acid production and development of bio dry mass (BDM), optical density and glucose (g/l) during cultivation of *C. glutamicum* KY10574 in F1 minimal medium, **40 g/l glucose**, 30°C, 25 ml medium in a 250 ml shake flask vessel (3 baffles); inoculated directly from BY agar plates; shaking rate: 150 rpm (0 – 45 h), 180 rpm (ca. 45 – 72 h), 210 rpm (≥ 72 h); amino acid analysis with GC.

4.4.3.3 25 ml cultivation (250 ml shake flask) with 60 g/l glucose

The cultivation results with 60 g/l of glucose in the F1 minimal medium are shown in Figure 4.21. Both the bio dry mass development and the amino acid production were successfully increased, especially the L-methionine concentration increased considerably. After 50 h more than 700 mg/l were produced and even in the following time under glucose limited conditions (see Figure 4.21) the concentration increased to values near 900 mg/l after 120 h. The reason could be the release of intracellular accumulated L-methionine in the supernatant after the cell wall collapse due to nutrient deficiency. This effect could also be observed for L-valine (maximum value: 300 mg/l after 120 h) and glycine (maximum value: 460 mg/l after 120 h). The course development for L-alanine was different, the maximum production was located after 46 h with 300 mg/l; afterwards the characteristical decrease could be observed. The maximum values of all amino acids shown in Figure 4.21 increased compared to the cultivation experiments with lower glucose levels at start (Figure 4.20 and 4.19).

The bio dry mass reached levels higher than 14 g/l. In contrast to the cultivation with 40 g/l glucose in the medium a very efficient transfer of enhanced cell growth (which was reached through the increase from 20 to 40 g/l of glucose in the medium) to a noticeable enhancement of the observed production of all amino acids could be seen.

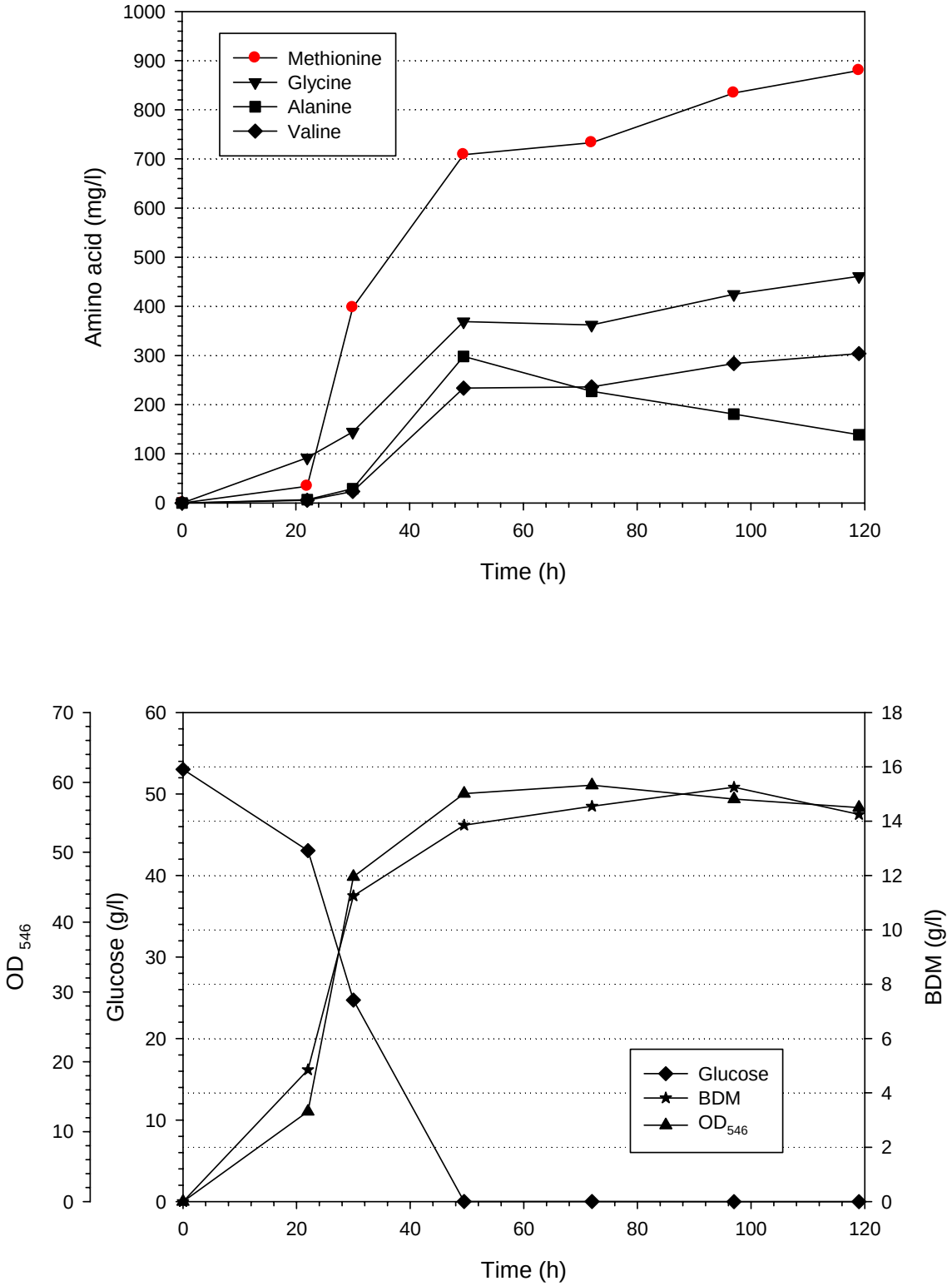


Figure 4.21: Amino acid production and development of bio dry mass (BDM), optical density and glucose (g/l) during cultivation of *C. glutamicum* KY10574 in F1 minimal medium, **53 g/l glucose** (value by analysis), 30°C, 25 ml medium in a 250 ml shake flask vessel (3 baffles); inoculated directly from BY agar plates; shaking rate: 150 rpm (0 – 45 h), 180 rpm (ca. 45 – 72 h), 210 rpm (≥ 72 h); amino acid analysis with GC.

4.4.3.4 25 ml cultivation (250 ml shake flask) with 80 g/l glucose

The cultivation with a glucose content of 80 g/l at the beginning featured surprising results, as shown in Figure 4.21. As it has been indicated before the L-alanine concentration increased with higher glucose levels but in this case the production of L-alanine came to pass to the disadvantage of the amino acids L-methionine and glycine (compare with Figure 4.21). L-alanine was obtained in amounts of approximately 600 mg/l without further concentration decrease (Figure 4.30). L-methionine was gained in amounts of nearly 500 mg/l, glycine concentrations achieved levels of ca. 250 mg/l. Another difference was the appearance of L-glutamic acid at 300 – 400 mg/l. This amino acid was produced at very low amounts in former cultivations using lower glucose concentrations in the medium. With a constant concentration around 300 mg/l L-valine was the sole exception. Compared to Figure 4.21 no obvious fluctuation took place.

The glucose was not consumed before 72 h due to the high provided concentration at the beginning and the values for the BDM (approx. 15 - 16 g/l) and the OD_{546} were in normal ranges.

4.4.3.5 25 ml cultivation (250 ml shake flask) with 100 g/l glucose

The results obtained with 100 g/l glucose in F1 minimal medium are shown in Figure 4.23. The amino acid production patterns which were obtained in this case were similar to those in the experiments with 80 g/l. There were only minor alterations in the product amounts compared to Figure 4.22. The highest production of any amino acids is L-alanine with 625 mg/l at the maximum, followed by L-methionine (ca. 500 mg/l), L-valine (ca. 330 mg/l), glycine (ca. 270 mg/l) and L-glutamic acid (ca. 235 mg/l). The L-methionine production finished after 47 h. The glucose was not consumed totally and the values settled down after 100 h at 10 g/l. This indicated a glucose excess supply and the ocurrence of the limitation of other nutrient components (e.g. nitrogen, sulphur or phosphate) in the cultivation. L-alanine was not degraded to intermediates of the citric acid cycle; due to the glucose excess there was no necessity to form new glucose molecules from amino acids for the metabolism. In accordance with that the values for biomass and OD_{546} finished on a level known from the cultivation reported before.

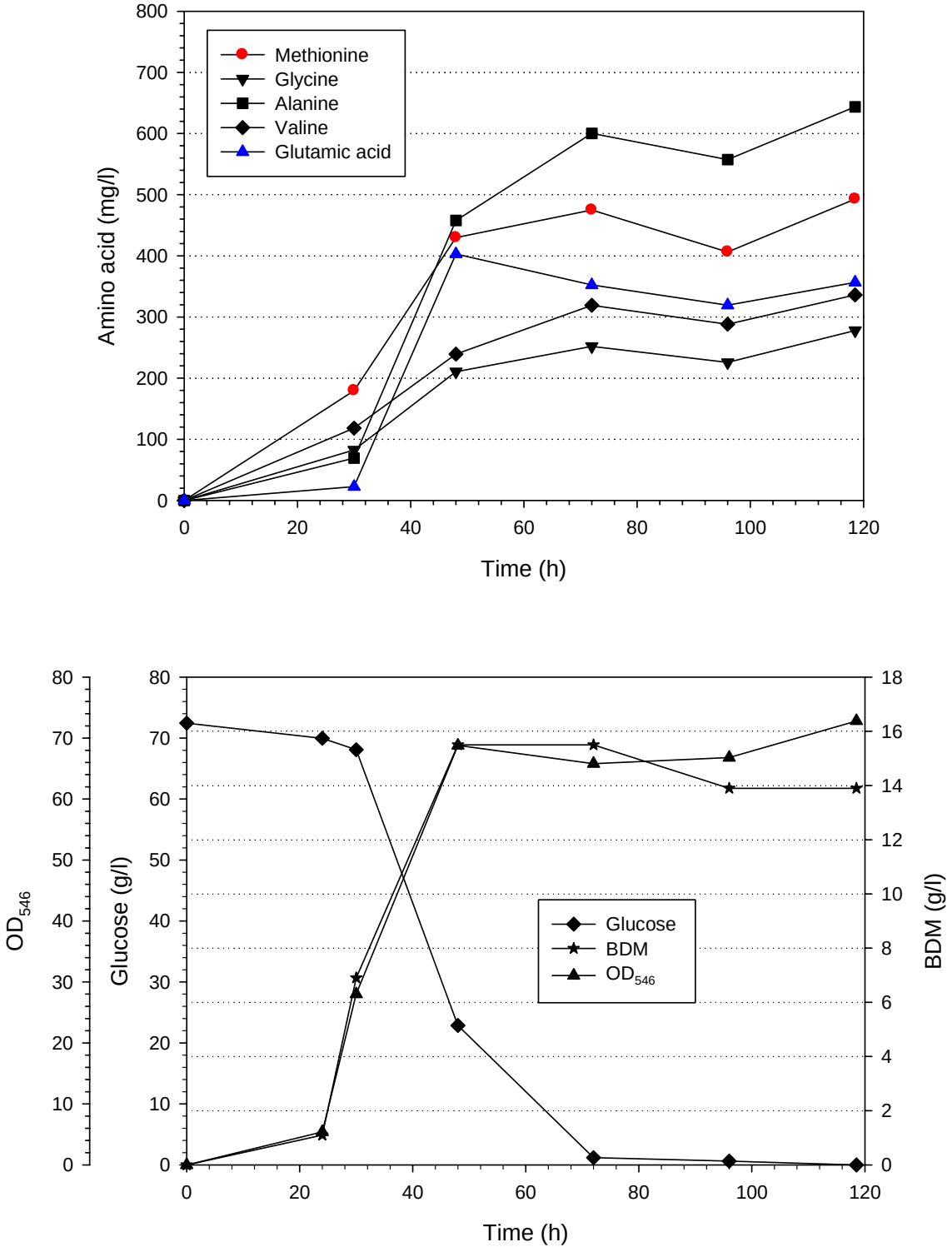


Figure 4.22: Amino acid production and development of bio dry mass (BDM), optical density and glucose (g/l) during cultivation of *C. glutamicum* KY10574 in F1 minimal medium, **73.5 g/l glucose** (value by analysis), 30°C, 25 ml medium in a 250 ml shake flask vessel (3 baffles); inoculated directly from BY agar plates; shaking rate: 150 rpm (0 – 45 h), 180 rpm (ca. 45 – 72 h), 210 rpm (≥ 72 h); amino acid analysis with GC.

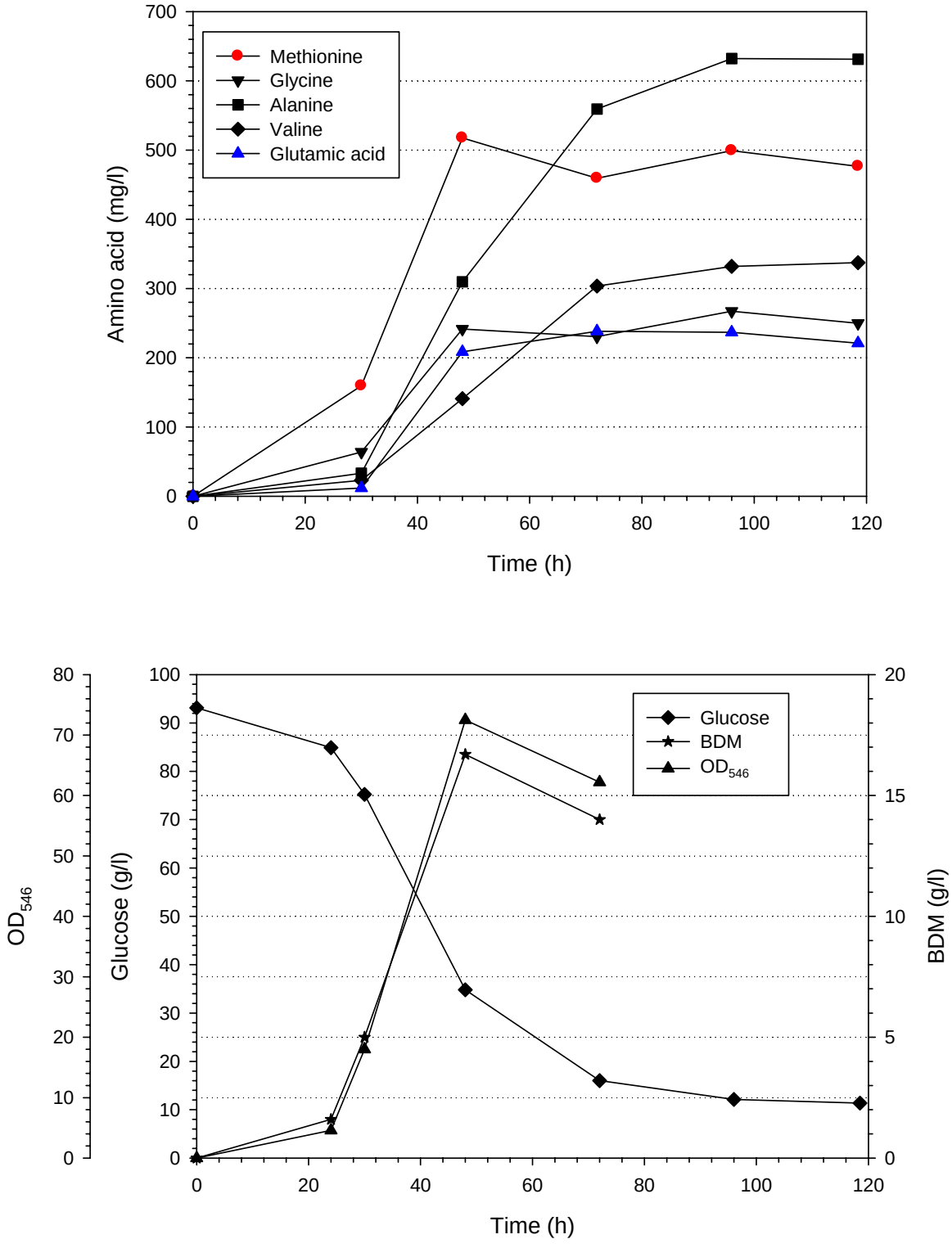


Figure 4.23: Amino acid production and development of bio dry mass (BDM), optical density and glucose (g/l) during cultivation of *C. glutamicum* KY10574 in F1 minimal medium, **95 g/l glucose** (value by analysis), 30°C, 25 ml medium in a 250 ml shake flask vessel (3 baffles); inoculated directly from BY agar plates; shaking rate: 150 rpm (0 – 45 h), 180 rpm (ca. 45 – 72 h), 210 rpm (≥ 72 h); amino acid analysis with GC.

4.4.3.6 100 ml cultivation (1 L shake flask) with 60 g/l glucose

The highest amount of L-methionine in the 250 ml shake flask scale (3 baffles) with 25 ml F1 minimal medium was gained with 60 g/l glucose as carbon source at the beginning of the cultivations. All experiments were also performed with 1 L shake flasks (2 baffles) and 100 ml medium. Also, the shaking rates were altered in the same way: 150 rpm (0 – 45 h), 180 rpm (ca. 45 – 72 h), 210 rpm (since ca. 72 h). The comparable results for the cultivations with a glucose level of 53 g/l with 100 ml medium in a 1 L shake flask are shown in Figure 4.24. The differences become clear by comparing the produced amounts of L-methionine as shown in Figure 4.24 with the amounts shown in Figure 4.21 using the smaller cultivation scale. L-Methionine was produced in maximum of 400 mg/l after 96 h, the other amino acids were also diminished in their concentration values.

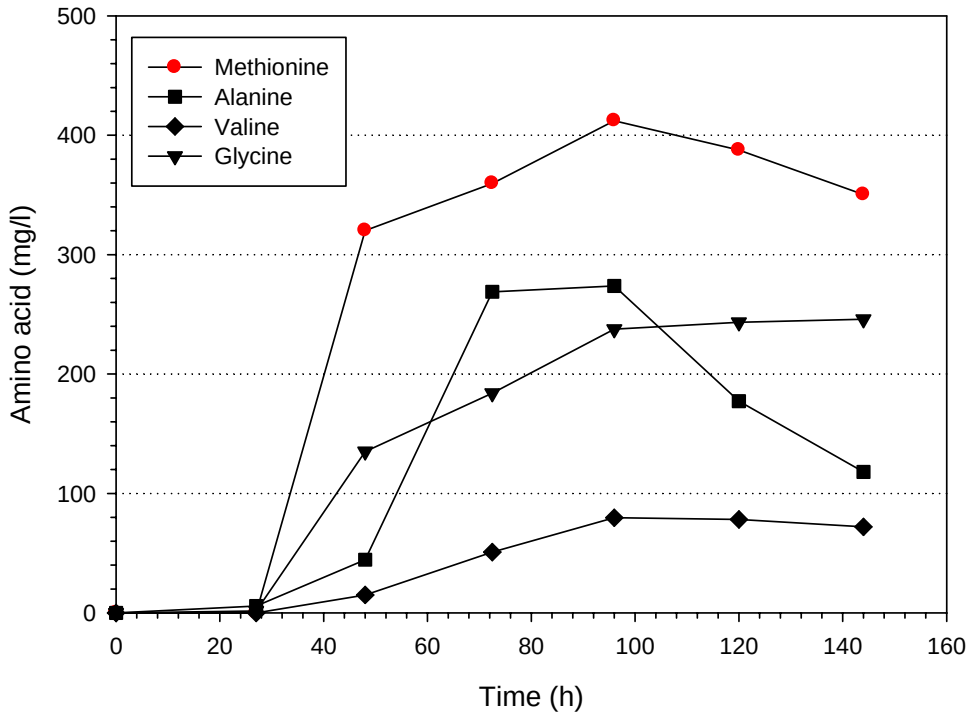


Figure 4.24: Amino acid production during cultivation of *C. glutamicum* KY10574 in F1 minimal medium, **53 g/l glucose** (value by analysis), 30°C, 100 ml medium in a 1 L shake flask vessel (2 baffles); inoculated directly from BY agar plates; shaking rate: 150 rpm (0 – 45 h), 180 rpm (ca. 45 – 72 h), 210 rpm (≥ 72 h); amino acid analysis with GC.

It was obvious that there were significantly lower production levels of amino acids in all experiments with the bigger scale cultivation. The reason for that was most probably the insufficient oxygen supply which could not be guaranteed in this shake flask scale using 100 ml me-

dium. Another reason for reduced mixing and oxygen supply could be also the lower number of baffles (2) in 1 the 1 L shake flasks compared to the 250 ml shake flasks (3). Consequently, *C. glutamicum* did not produce the amino acids derived in the lower scale experiments (Gomes et al. 2005). The major influence of oxygen supply on amino acid production with *C.glutamicum* KY10574 was also clearly visible in the bioreactor experiments (chapter 4.4.6). Therefore, there is a comparison of the results obtained from both scales in Figure 4.25.

4.4.3.7 Comparison of the L-methionine production with *C.glutamicum* KY10574 with different glucose amounts and shake flask scales

The completion of chapter 4.4.3 with its series of experiments on the influence of the carbon source (glucose) and the rising shaking rates (150 – 210 rpm) in 2 different shake flask scales is presented in Figure 4.25. The highest level of L-methionine production (900 mg/l after 119 h) could be observed with 60 g/l glucose at the beginning of the smaller cultivation scale. Concentrations around 500 mg/l of L-methionine were obtained in the small shake flask scale with glucose concentrations around 60 g/l. It was not expedient to use smaller amounts than 40 g/l in the medium because of the fast glucose consumption and subsequently the end of cell growth due to carbon source limitation. In the bigger shake flask scale the L-methionine level reached values which were only near 500 mg/l at the maximum (using 60 + 80 g/l glucose in medium). Obviously, there was a limitation in oxygen supply but there was no way to increase the shaking rate speed above 210 rpm due to the massive splashing of the liquid at these velocities. A problem in all shake flasks was a substantial layer of foam on the surface of the cultivation broth which impeded the transport of oxygen in the interior areas of the liquid. The foam was also one of the biggest problems in the bioreactor cultivations (chapter 4.4.7).

The results from Figure 4.25 represent a summary of an exemplary experimental series (Figure 4.19 – 4.23) which is very characteristical for amino acid production with *Corynebacterium glutamicum* KY10574. But the experiments were carried out with the same conditions several times. The average of L-methionine production values in all shake flasks which were performed under the same conditions with shaking rates from 150 – 210 rpm and different glucose amounts are shown in Figure 4.26. There are some slight alterations in the L-methionine production level compared to Figure 4.25. The highest concentrations were reached using 60 g/l of glucose as carbon source in the small scale (25 ml medium in 250 ml

shake flasks) and 80 g/l of glucose in the big scale (100 ml medium in 1 L shake flasks). In general, the L-methionine values were higher using glucose values between 70 and 100 g/l than values between 20 and 40 g/l. Glucose levels under 50 g/l were consumed very quickly and caused an early amino acid production stop. The product spectrum of amino acids can be altered through minor changes in parameters like cell growth, pH and shaking rates and medium composition. Additionally the strain *C. glutamicum* KY10574 is able to produce a broad variety of amino acids due to its setting up through random mutagenesis by Kyowa Hakko. Therefore, marginally alterations, e.g. in the important phase at the beginning of the cell growth might change considerably the development of the product spectrum in each shake flask.

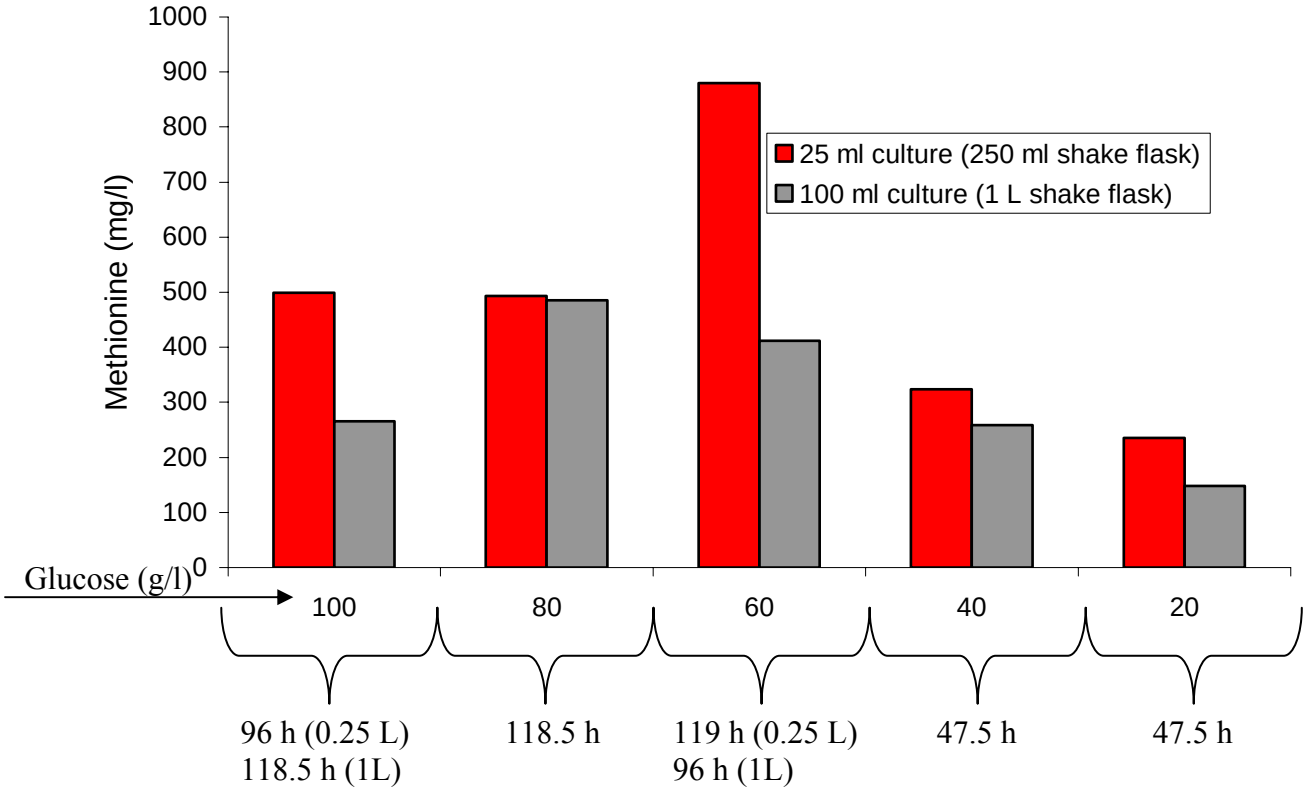


Figure 4.25: Comparison of the cultivations seen before on the influence of glucose amounts and 2 different shake flask scales (25 ml F1 medium in 250 ml shake flasks (3 baffles); 100 ml F1 medium in 1 L shake flasks (2 baffles) on the L-methionine production capability of *C. glutamicum* KY10574; shaking rate mode: 150 – 210 rpm (180 rpm since 45 h; 210 rpm since 72 h); temperature 30°C; amino acid analysis with GC. (The points in time when the L-methionine values were measured are indicated).

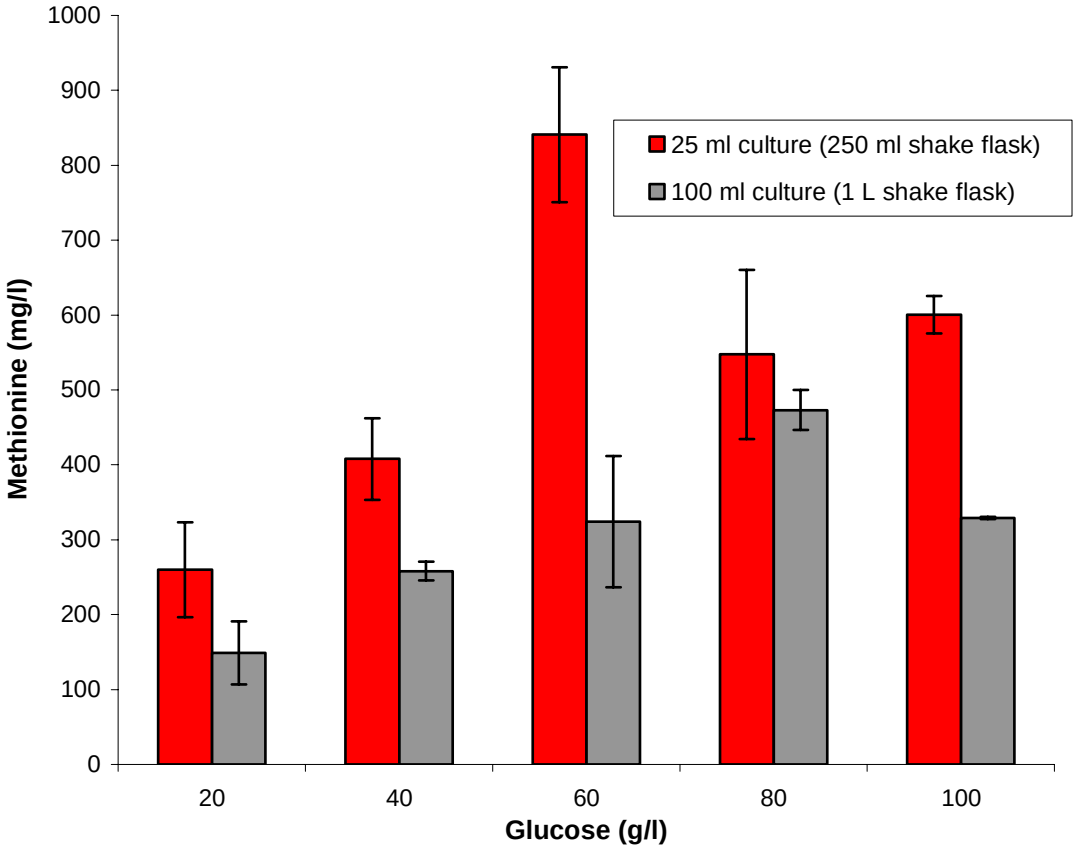


Figure 4.26: Average L-methionine production values in 2 shake flask scales in F1 minimal medium with varied glucose concentrations; 25 ml F1 medium in 250 ml shake flasks (3 baffles); 100 ml F1 medium in 1 L shake flasks (2baffles); temperature 30°C; shaking rate mode: 150 – 210 rpm (180 rpm since 45 h; 210 rpm since 72 h), amino acid analysis with GC.

After comparing the shake flask results in both scales the wide variety of produced amino acids in several concentration ranges became more and more obvious. The strain *C. glutamicum* KY10574 was derived through random mutagenesis experiments (see chapter 2.5). A characteristic feature of this method is the appearance of alterations in several biochemical pathways, not only in the favourised target pathways. This attribute leads inevitably to the deregulation of several production pathways, subsequently the feedback repression and inhibition signals of many amino acids are also partially deactivated. So the strain KY10574 was developed in order to get a methionine overproducer, but other mutations could not be avoided which lead to the production of other amino acids as by-products after the alteration of their regulation mechanisms (Kumar et al. 2005). The directed mutation of the biochemical pathways of amino acids in *C. glutamicum* by genetic engineering has the major advantage that unrequested mutations in other biochemical pathways than the desired one can be avoided completely (Wittmann et al. 2004).

4.4.4 Shake flask cultivation of *C. glutamicum* KY10574 in F1 medium with different shaking rates

The Figure 4.27 shows the differences in L-methionine production using several shaking rates. The highest possible shaking rate at the beginning could be 120 or 150 rpm due to the extensive splashing of the liquid medium. The shaking rate could be enhanced after 45 h (180 rpm) and 72 h (210 rpm), because the density and viscosity of the medium was much higher due to the accumulation of biomass. The enhancement of the shaking rate in order to enhance the L-methionine concentration was successful for the smaller scale (25 ml in 250 ml flasks). Velocities of 150 rpm were absolutely necessary to get higher L-methionine production concentrations than 500 mg/l. The improvement of the oxygen supply using a shaking rate of 150 rpm was clearly visible for the smaller scale. Higher shaking rates could increase the production only marginally. In the case of the bigger scale higher shaking rates showed no influence. Obviously it was much more difficult to supply the microorganisms with sufficient amounts of oxygen in this shake flask scale.

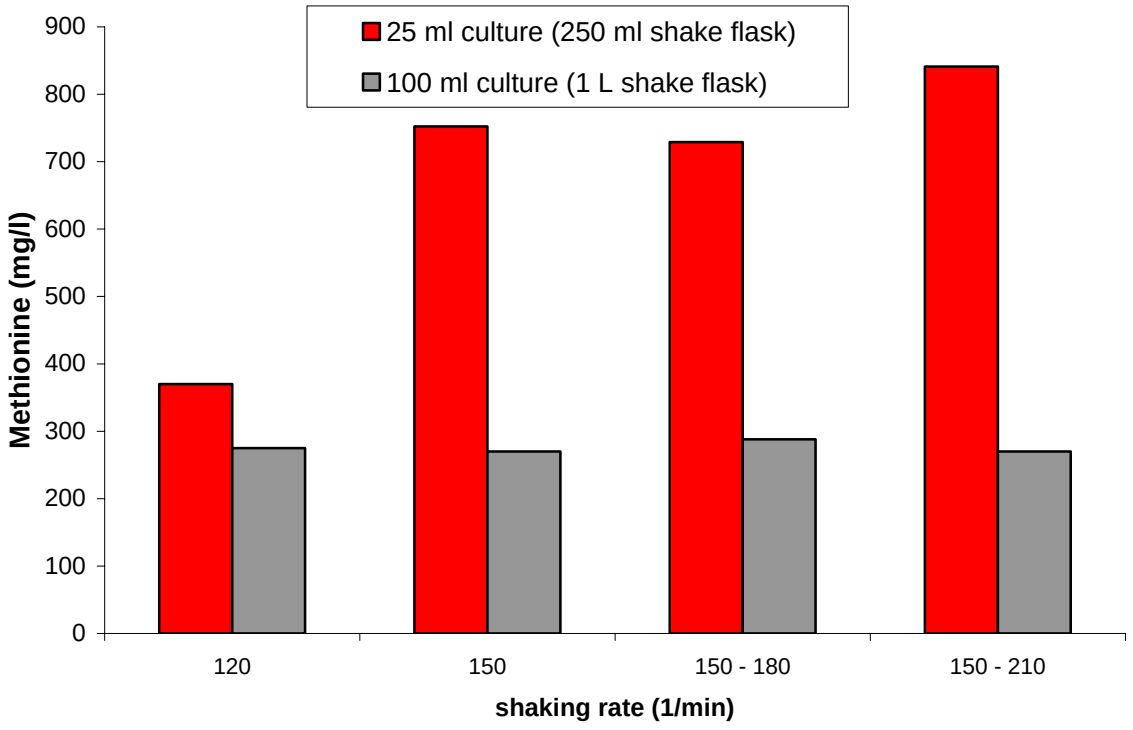


Figure 4.27: L-methionine production values in 2 shake flask scales using **different shaking rates from 120 – 210 rpm** (25 ml F1 medium in 250 ml shake flasks (3 baffles); 100 ml F1 medium in 1 L shake flasks (2 baffles)); temperature 30°C; glucose 60 g/l, amino acid analysis with GC.

4.4.5 Shake flask cultivation in F1 minimal medium using precultures

The adaptation of the microrganisms to the minimal medium F1 was an important factor. This was a major problem in the first cultivations; in every second shake flask cultivation the microorganisms had problems to grow. In order to avoid the long transition phase for biomass growth at the beginning and to enhance the amino acid production precultures were used.

Table 4.1 shows the concentrations of 4 amino acids after 48 h of shake flask cultivation. The glucose concentration (56 g/l) was optimised according to the results obtained in former experiments (see Figure 4.25), as well as the shaking rate mode (see Figure 4.27). This cultivation broth was the preculture for 2 shake flask experiments with the same minimal medium (Figure 4.28, inoculated in a ratio of 1:10). These shake flask experiments were carried out with the same conditions as in the preculture. In both shake flask experiments, which are presented in Figure 4.28, the L-methionine formation increased remarkably to values above 1.2 g/l in the first 42 h of the cultivations. There was no lag-phase for microbial adaptation at the beginning of the cultivation after inoculation. So the microorganisms could use the available nutrients in a very fast and efficient way which allowed a production enhancement to 1.4 g/l (average value of both cultivations) after 144 h.

It was also noticeable that the production of L-methionine even at the end of the cultivation time still increase a little bit which could be also observed in Figure 4.21. A possible reason might be that at this point in time the microbial cell walls began to collapse caused by nutrient deficiency and released intracellular amounts of L-methionine (accumulated in the cytosol) into the extracellular area. This could promote the L-methionine production even under insufficient nutrient supply conditions.

Table 4.1: Amino acid production during cultivation of *C. glutamicum* KY10574 in F1 medium, **56 g/l glucose**, 30°C, 25 ml medium in a 250 ml shake flask vessel (3 baffles); inoculated directly from BY agar plates; **shaking rate mode: 150 rpm (0 – 45 h), 180 rpm**; amino acid analysis with GC. This culture was used as preculture for the cultivation shown in Figure 4.28.

Time (h)	L-Methionine (mg/l)	L-Alanine (mg/l)	Glycine (mg/l)	L-Valine (mg/l)
27	25	16.3	16.4	4.6
48	658.2	355.3	280.3	563

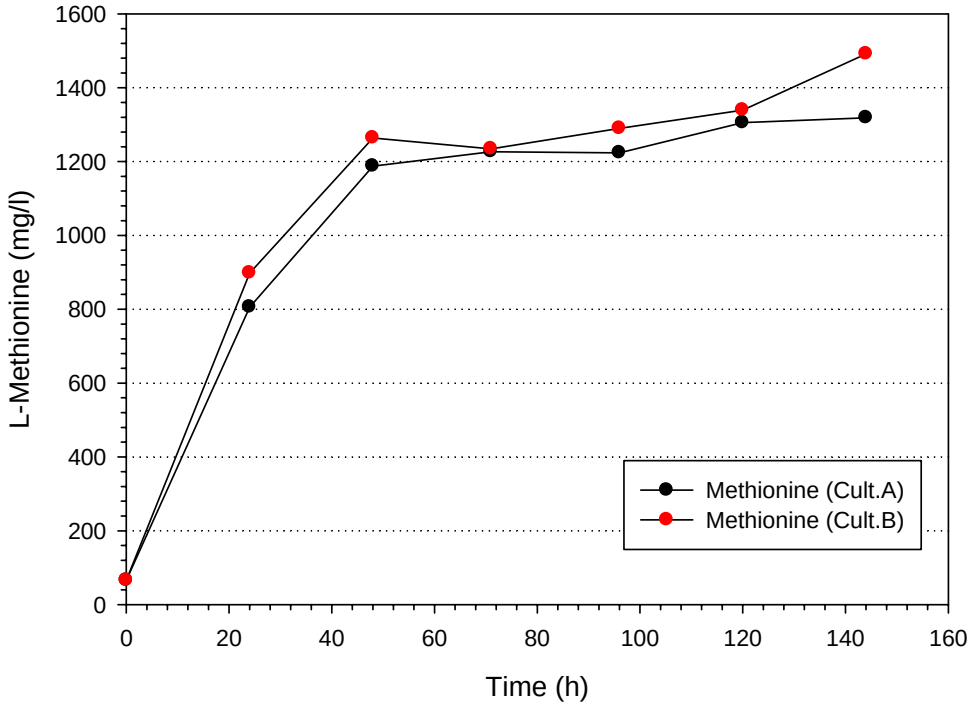


Figure 4.28: Amino acid production during cultivation of *C. glutamicum* KY10574 in F1 medium, **56 g/l glucose,** 30°C, 25 ml medium in a 250 ml shake flask vessel (3 baffles); inoculated with 2.5 ml from preculture (shown in Table 4.1); **shaking rate mode: 150 rpm (0 – 45 h), 180 rpm**; amino acid analysis with GC.

4.4.6 Shake flask cultivation of *Corynebacterium glutamicum* KY10574 using metabolic precursors of L-methionine

The influence of the metabolic precursors of L-methionine on the production of the amino acid with the strain *C. glutamicum* DSM20300, L-homoserine and L-homocysteine, were shown before in chapter 4.2.2 (Figure 4.4). Homocysteine is the last metabolic precursor on the biochemical pathway to L-methionine formation (see Figure 2.11), whereas homoserine is a corporate precursor of the amino acids lysine, threonine and methionine (Voet et al. 2002). The influence of the addition of 0.5 g/l of these precursors into the cultivation medium F1 was investigated as shown in Figure 4.29 comparing it to a cultivation without these precursors. According to these data it can be concluded that no improvement of the L-methionine production could be observed. On the contrary, the cultivation without any precursors showed the maximum L-methionine production.

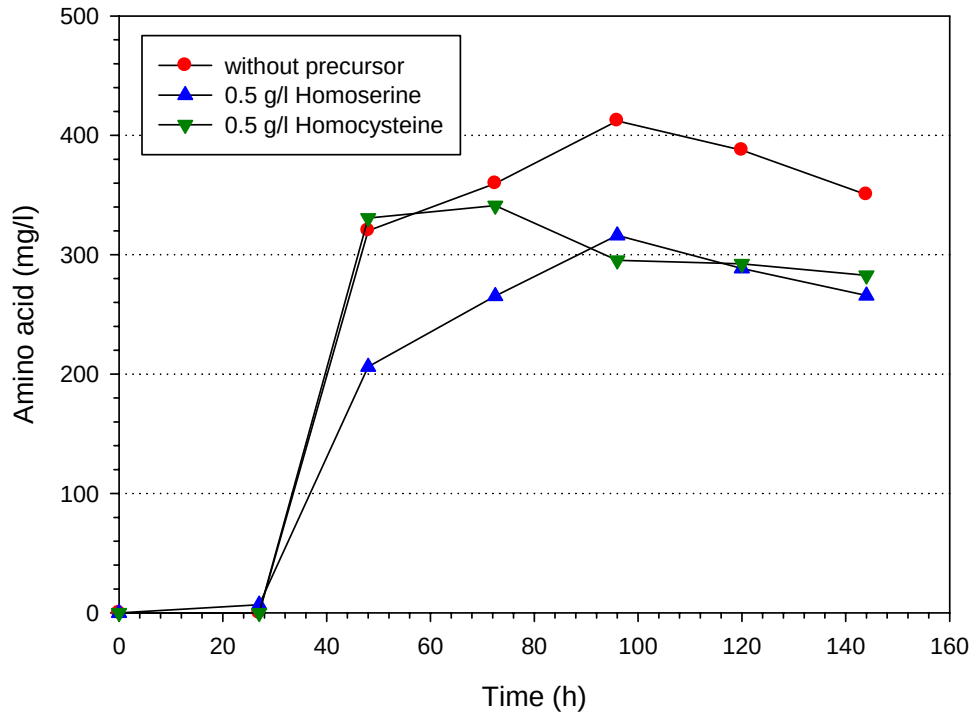


Figure 4.29: Comparison of *C. glutamicum* KY10574 cultivations with and without the addition of 0.5 g/l homoserine and homocysteine in F1 minimal medium with **40 g/l glucose**; 100 ml medium in 1 L shake flasks (2 baffles), directly inoculated from BY agar plates; 30°C; shaking rate: 150 rpm (0 – 45 h), 180 rpm; 53 g/l glucose; amino acid analysis with GC.

The inhibition mechanisms maintaining feedback repression and inhibition phenomenons (Figure 2.10) which are responsible for deactivating the L-methionine pathway through the addition of high amounts of the precursors could not be prevented successfully. The effect seemed to bring about quite the contrary. The control mechanisms, which were altered in the strain *C.glutamicum* KY10574 at least partially because this strain was actually able to over-produce L-methionine, got again more influence on production prevention by additional adding of homoserine and homocysteine.

By direct comparison of the progression of the curves for the cultivations with homoserine and homocysteine the faster production using homocysteine seemed remarkable. But at the end of the cultivation time both cultivations showed almost the same L-methionine concentration.

4.4.7 Cultivation of *Corynebacterium glutamicum* KY10574 in the bioreactor scale (3.5 L)

The bioreactor experiments were performed in bioreactors of the company Infors in a 3.5 L scale. It was possible to control the aeration rate [0.5 L/(L • min)] and the stirring speed in combination with the oxygen partial pressure (pO_2). If the pO_2 dropped under 20% the stirring speed was enhanced automatically to guarantee a better oxygen supply in the cells by higher mixing rates, which was much more effective than the enhancement of the aeration rate. The bioreactor cultivation was performed with glucose as the limiting factor to be able to control growth. This is the state of the art method to cultivate strains of *Corynebacterium glutamicum* in the bioreactor scale in order to be able to control cell growth and avoid substrate inhibition and foaming problems (Kumar et al. 2003; Eggeling et al. 1999). For this reason, the glucose concentration at the beginning was only around 10 – 20 g/l, the glucose feed (2 • 200 ml; 300 g/l) started immediately after the moment when the glucose was consumed with a distinct pumping rate to guarantee the carbon source supply (fed batch process).

In the following chapter 4 bioreactor cultivations will be shown (in chronological order) to demonstrate the progress in L-methionine production, the problems (foaming!) and additional options in the process controlling. Cultivation parameters like aeration rate, pH, foam development and glucose feed could be adjusted very precisely in comparison to the shake flasks. In all cases the the bioreactor cultivations were inoculated with 200 ml of shake flask precultures which were performed for 48 h at 30°C with 150 rpm (F1 minimal medium, 100 ml in 1 L shake flasks). So there were new approaches to influence the development of cell growth and L-methionine production in the bioreactor.

4.4.7.1 Bioreactor cultivation with 10 g/l glucose at t = 0 and a continuous feeding strategy for additional glucose with high pumping rates

The first bioreactor cultivation with the strain *C. glutamicum* KY10574 was performed with an amount of 10 g/l of glucose in the cultivation medium (F1 medium, see Table 3.6). 2.8 L medium were inoculated with 200 ml of a shake flask preculture. Additionally, a glucose feed [2 • 200 ml, 300 g/l, pumping rate mode: 70 ml/h (11 h-14 h), 15 ml/h (since 14 h)] of 400 ml and 100 ml of anti-foam substances were added to the bioreactor. Altogether 150 g glucose were given to the cultivation. Figure 4.30 describes the development of the production of the

amino acids glycine and L-methionine as well as the development of cell growth (BDM) and glucose consumption. Only two amino acids, glycine and methionine, were produced in remarkable amounts, as shown in graph. The biomass increased rapidly to values of 16 g/l after 24 h, which was not reached in any shake flask experiment.

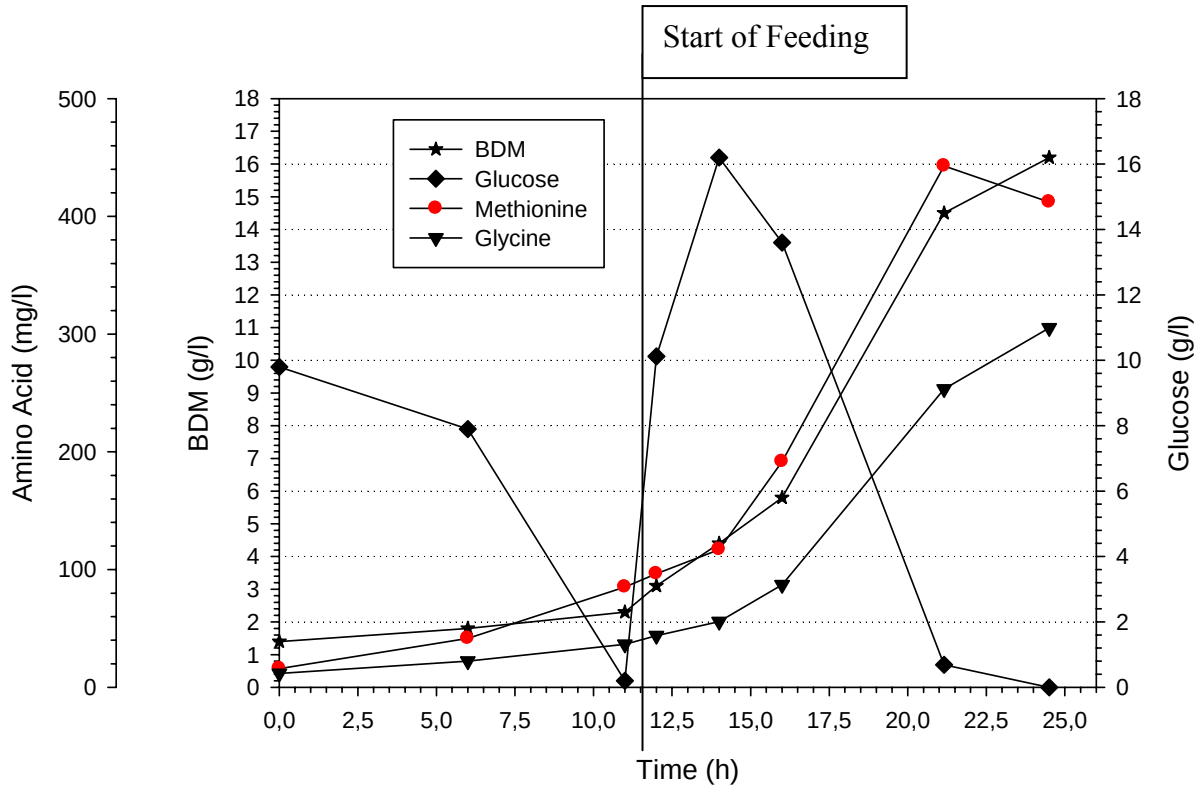


Figure 4.30: Bioreactor cultivation (3.5 L) of *C. glutamicum* KY10574 over 25 h at 30°C in F1 minimal medium with 10 g/l glucose (t = 0); glucose feed (2 • 200 ml, 300 g/l), pumping rate mode: 70 ml/l (11h-14h), 15 ml/h (since 14h); presented are the courses for the development of the BDM, the glucose and the produced amino acids L-methionine and glycine; inoculation with 200 ml preculture (F1 medium) from shake flask cultivation; aeration rate: 0.5 L/(L • min); amino acid analysis with GC.

The fast cell growth velocity and biomass accumulation were also a major disadvantage and problem, because an extreme foaming of the cultivation broth occurred after 24 h which could not be reduced effectively through the addition of anti-foam reagent (Struktol J673, diluted 1:10 with H_2O). Foam formation could be detected by a foam sensor which was automatically connected with pumps for anti-foam reagent. The experiment was finished after 24 h because the bioreactor began with overfoaming. The cell growth was too fast and not controllable in this phase of the cultivation. The main reason for this development was the glucose level. As shown in Figure 4.30, the glucose pool (the initially supplied sugar) was exhausted after 11 h and the glucose feed was performed afterwards with much too high pumping rates. This led to the accumulation of the carbon source in the medium and consequently, there was no limita-

tion of cell growth any more. The potential of the microorganisms to produce L-methionine and glycine under these conditions was obvious, within 24 h 425 – 450 mg/l L-methionine and 300 mg/l glycine were obtained. These results suggest that even much higher yields of those amino acids are feasible in cultivations which are performed over longer time periods.

The impacts of glucose accumulation in the medium and the absence of a limitation factor on the oxygen supply are presented in Figure 4.31. The oxygen partial pressure (pO_2) dropped under 20% and the stirring speed was increased automatically to the adjusted limit of 950 rpm in order to enhance the oxygen supply inside the cultivation broth. Despite the acceleration of the stirring speed the decrease of the pO_2 values could not be prevented. The increase of the pO_2 during the last hour (Figure 4.31) occurred even after the cultivation began with over-foaming. Stirring speed and aeration rate were enhanced in order to avoid the oxygen limitation but they also provoked more foam formation.

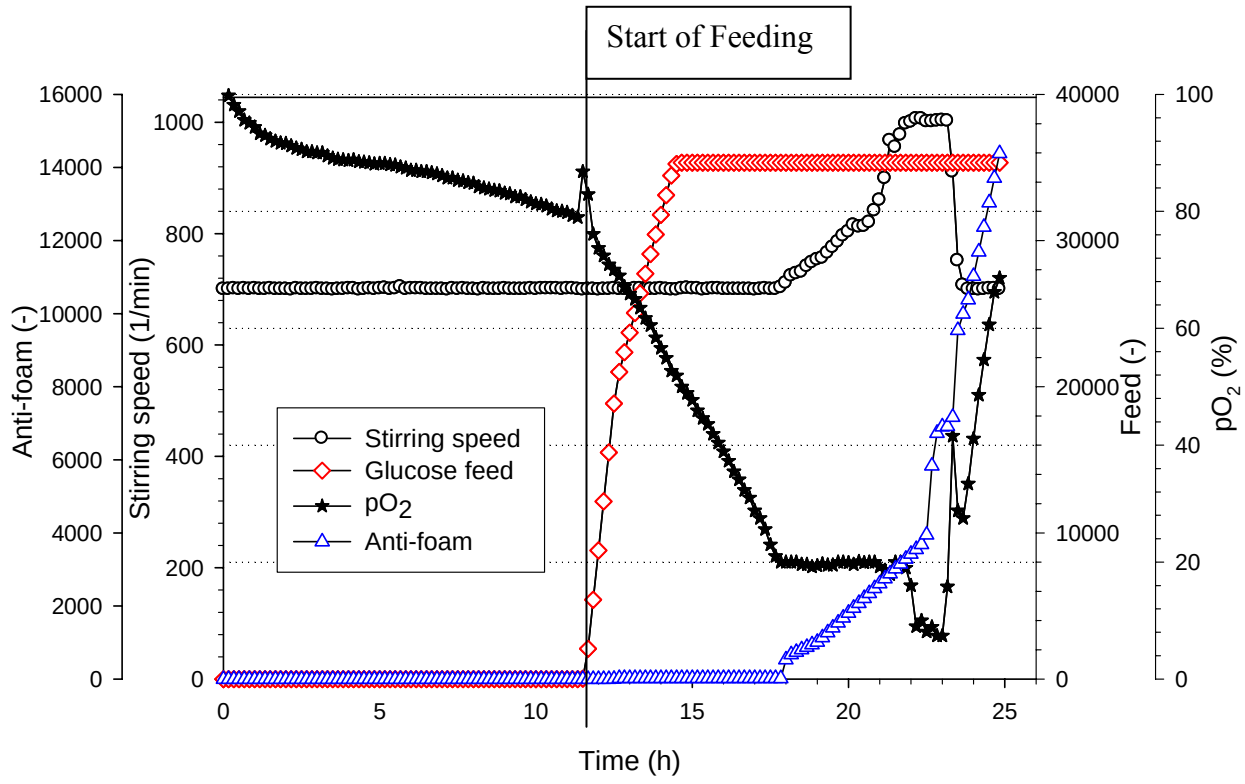
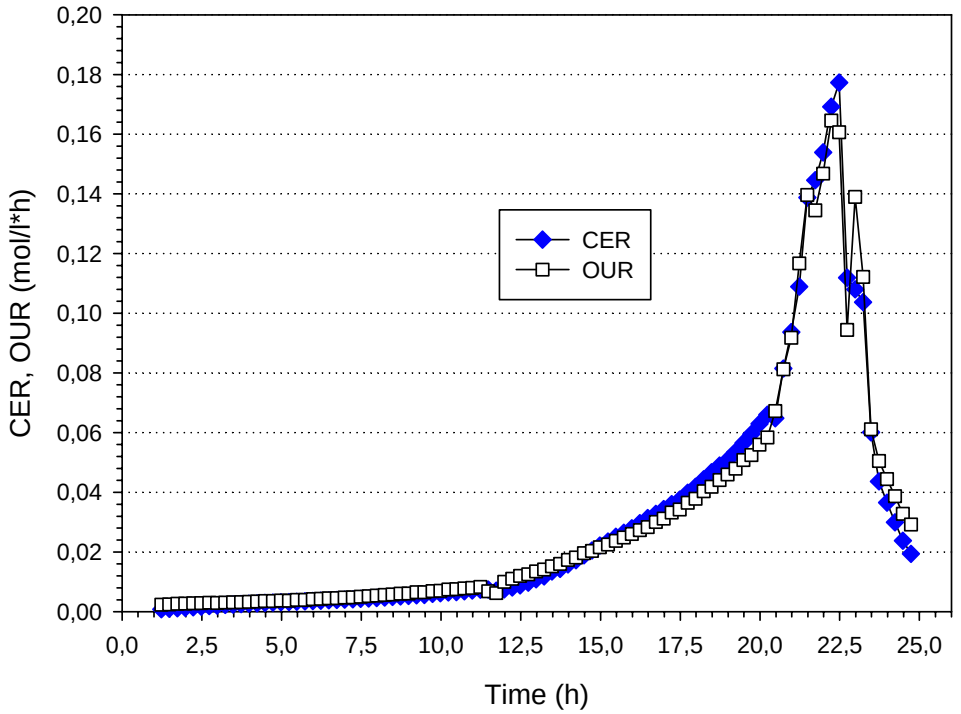


Figure 4.31: Bioreactor cultivation (3.5 L) of *C. glutamicum* KY10574 over 25 h at 30°C in F1 minimal medium with 10 g/l glucose (t = 0); presented are the courses for the development of the pO_2, the stirring speed and the pump rates [70 ml/h (11-14h), 15 ml/h (since 14h)] for the addition of the glucose feed (300 g/l, 2 • 200 ml) and the anti-foam substance; inoculation with 200 ml preculture (F1 medium) from a shake flask cultivation; aeration rate: 0.5 L/(L • min).

Figure 4.31 shows also the pumping rates for the addition of the anti-foam reagent and the glucose (Feed). According to the slow cell growth in the first 11 h of the cultivation (see Figure 4.30) glucose was not added continuously to the bioreactor but added all at once. The effects of these crucial occasions on the cell growth and the foam formation had been clearly underestimated as Figures 4.31 and 4.30 show. The overproportional increase of the addition of anti-foam reagent at the end of the cultivation could not prevent the strong enhancement of foam development in the bioreactor.

The exhaust gas analysis is an indicator for the potential of the microorganisms to utilize oxygen from fresh air and to produce carbon dioxide, it is a valuable tool for determination of metabolic activity. The courses for the development of the oxygen uptake rate (OUR) and the carbon dioxide evolution rate (CER) are shown in Figure 4.32. This Figure shows also the respiratory coefficient (RQ). The respiratory coefficent RQ is calculated as the coefficient q_{co2} / q_{o2} (referring to molar mass). In Figure 4.32 the connection between the oxygen uptake rate (OUR) and the carbon dioxide formation rate became clearly obvious. The courses were nearly parallel; if the OUR course increased, than the CER course developed parallel and rose too. The potential to transfer oxygen to CO_2 was enhanced if there was more available oxygen for utilization. The big rise of the courses at the end of the cultivation (maximum at 21,5 h) can be referred to the exponential cell growth in this time period of cultivation (see Figure 4.30). According to the higher oxygen demand of the microorganisms due to their fast proliferation (uncontrolled growth) and the increased stirring speed the values for OUR and CER rose overproportionally. The values fell down again after the bioreactor cultivation began with overfoaming, the outlet for exhaust gas analysis was partially blocked with liquid contents.

The respiratory coefficient RQ shows a course development with values around 1. If the RQ values are below 1 more carbon dioxide is produced than oxygen is taken from fresh air. Values above 1 are indicators for the contrary. The course development in the first 5 h (see Figure 4.32) is not meaningful due to very low values for CER and OUR. In general the data from the exhaust gas analysis (Figure 4.32) have to be seen as a mathematical model which intends to reflect reality, but not as absolute values like e.g. concentration data for amino acids or the bio dry mass.



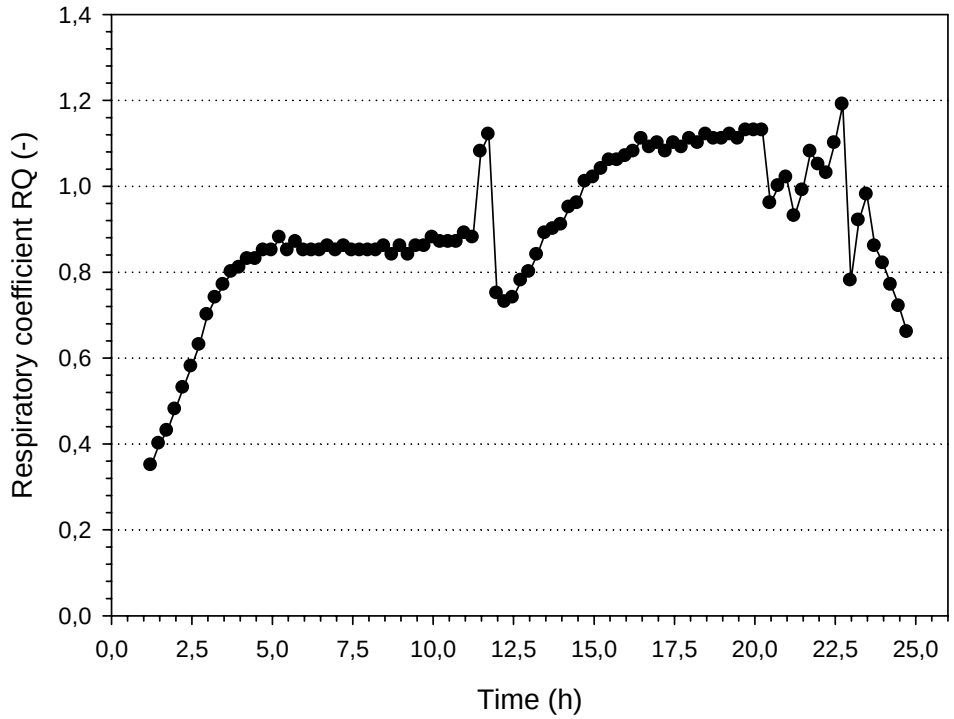


Figure 4.32: Course development of the oxygen uptake rate (OUR), the carbon dioxide evolution rate (CER) and the respiratory coefficient during the cultivation of *C. glutamicum* KY10574 in the bioreactor (3.5 L) in F1 minimal medium over 25 h at 30°C with 10 g/l glucose (t = 0); glucose feed (300 g/l, 2 • 200 ml), pumping rate mode: 70 ml/h (11 h-14 h), 15 ml/h (since 14 h); inoculation with 200 ml preculture (F1 medium) from shake flask cultivation; aeration rate: 0.5 L/(L • min).

4.4.7.2 Bioreactor cultivation with 10 g/l glucose at t = 0 and a continuous feeding strategy for additional glucose with low pumping rates

This bioreactor cultivation was performed under consideration of the results formerly obtained in the cultivation which was described in the chapter 4.4.7.1. The glucose concentration at the beginning was 10 g/l, the glucose feed was performed in difference to the first bioreactor cultivation with very low pumping rates (10 ml/h, 2 • 200 ml, 300 g/l). As shown in Figure 4.33, on the one hand, further controllable cell growth could be guaranteed and on the other hand glucose accumulation could be avoided. During the continuous feeding process the glucose concentration was located always nearly zero, therefore, controlled growth under limitation conditions was established. The bioreactor medium (2.8 L) was inoculated with 200 ml from shake flask preculture (48 h). In addition 400 ml glucose feed and 100 ml anti foam substances were given in the bioreactor during the cultivation time. During cultivation 150 g glucose were given altogether to the medium. In comparison to the first bioreactor cultivation (Figure 4.30) the cell growth slowed down; 11.5 g/l BDM were generated after 48 h. Amounts like these and more were achieved in the first cultivation already after 24 h but the bioreactor tended to foam over if the cell growth was too fast. Although the bio dry mass even after 48 h was lower than in the first cultivation after 24 h, these conditions are more favourable for the process because the problems related to foam began at a much later point in time (42 h, see Figure 4.34). The L-methionine and glycine concentrations increased continuously to values of 920 mg/l (methionine) and 500 mg/l (glycine) after 48 h towards the end of the cultivation.

After 48 h the cultivation was finished due to intense foaming and the overproportional addition of anti foam substance (see Figure 4.34). For this reason the amino acid production course stopped in maximum. In further cultivations over longer time periods it might be possible to obtain even higher concentrations of the amino acids methionine and glycine. But as a prerequisite, an effective process to reduce the foam has to be worked out.

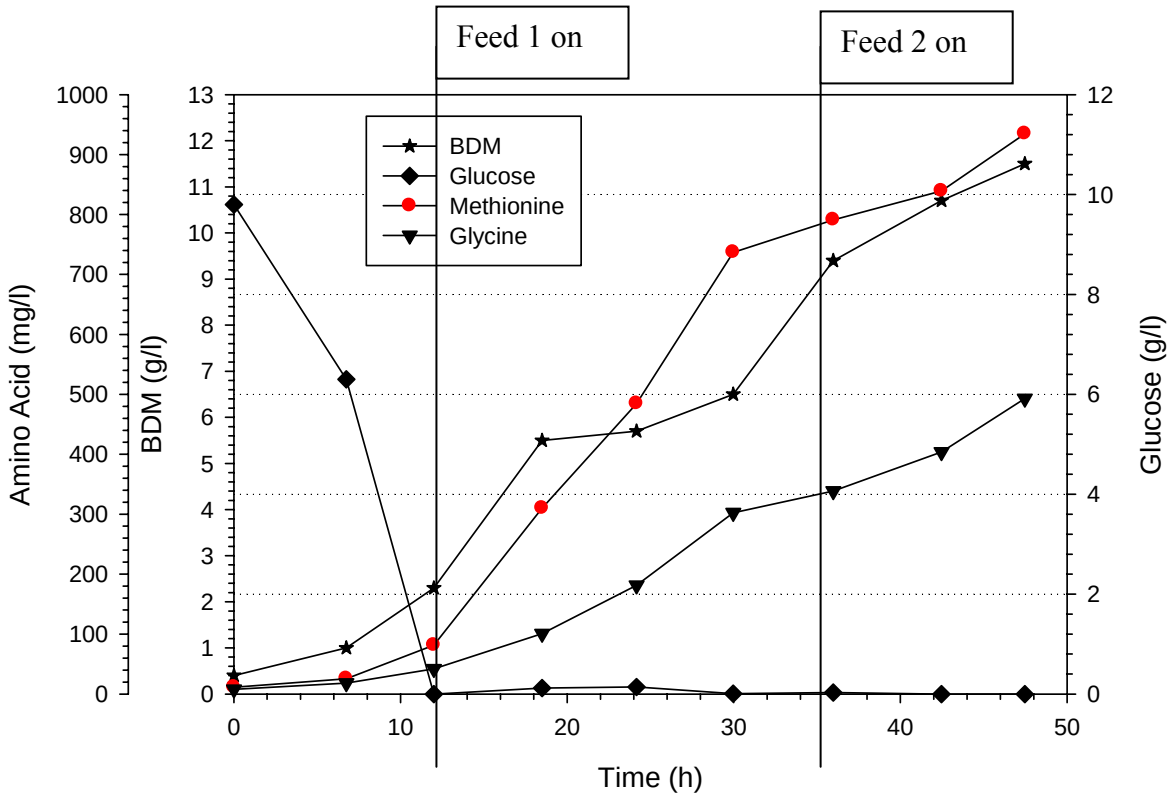


Figure 4.33: Bioreactor cultivation (3.5 L) of *C. glutamicum* KY10574 over 48 h at 30°C in F1 minimal medium with 10 g/l glucose (t = 0); glucose feed with a pumping rate of 10 ml/h (2 • 200 ml, 300 g/l), presented are the courses for the development of the biomass, the glucose and the produced amino acids L-methionine and glycine; inoculation with 200 ml preculture (F1 medium) from shake flask cultivation; aeration rate: 0.5 L/(L • min); amino acid analysis with GC.

It can be quite clearly stated that the glucose limitation factor after 12 h was the crucial cultivation, the low pumping rates for glucose feed addition made cell growth and product development more controllable although the foaming at the end of the cultivation was very strong. In contrast to the former experiment (Figure 4.30) with higher accumulated glucose concentrations and higher feeding rates (Figure 4.31) during the cultivation time, this time foaming could be reduced significantly. Not only that bioreactor cultivation is more controllable with one parameter responsible for limited growth, also the amino acid concentrations would be much more higher by distributing the glucose addition over longer time periods.

The development of pO_2, the stirring speed and the pumping rates for the addition of glucose and anti foam substance are shown in Figure 4.34. The pO_2 did not drop under 20% during the cultivation by a constant stirring speed of 700 rpm; so there were no phases of extreme anaerobic conditions with diminished cell growth and production in the bioreactor as it oc-

curred in former experiments (Figure 4.31). In comparison to experiments in the shake flasks there were no more remarkable amino acid side products like alanine or valine, this indicated that the production conditions for L-methionine and glycine were especially well adapted in the bioreactor. The strong foam formation (see Figure 4.34) towards the end of the cultivation could be evidence for another limitation by another nutrition source. However, the biomass growth curve was still increasing in this time period (Figure 4.33).

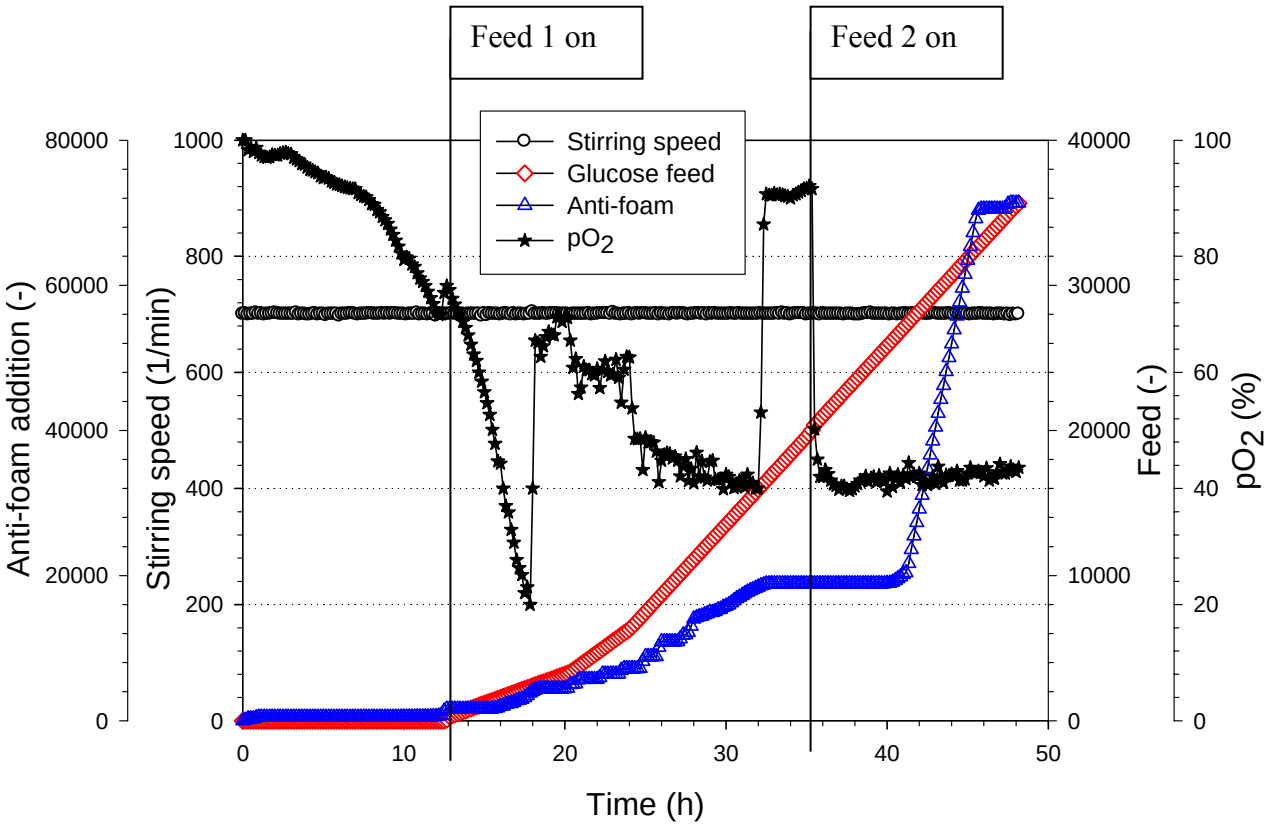


Figure 4.34: Bioreactor cultivation (3.5 L) of *C. glutamicum* KY10574 over 48 h at 30°C in F1 minimal medium with 10 g/l glucose (t = 0); presented are the courses for the development of the pO_2, the stirring speed and the pump rates for the addition of the glucose feed (10 ml/h, 300 g/l, 2 • 200 ml) and the anti-foam reagent (Struktol J673); inoculation with 200 ml preculture (F1 medium) from shake flask cultivation; aeration rate: 0.5 L/(L • min).

In Figure 4.35 the oxygen uptake rate (OUR) and the carbon dioxide formation rate (CER) are shown. The progressions of both curves interdepend strongly on each other. The big rise (maximum at 18h) and the big descent (minimum at 36 h) of the curve courses are referring to glucose limitation or absence (compare with Figure 4.32), the rise fits perfectly to the exponential cell growth phase (see Figure 4.33). Generally, the fluctuations of carbon dioxide formation (CER) were slightly higher. This parameter was more sensitive than the oxygen uptake rate (OUR) in the experiments and reacted very quickly to variations in the metabolism.

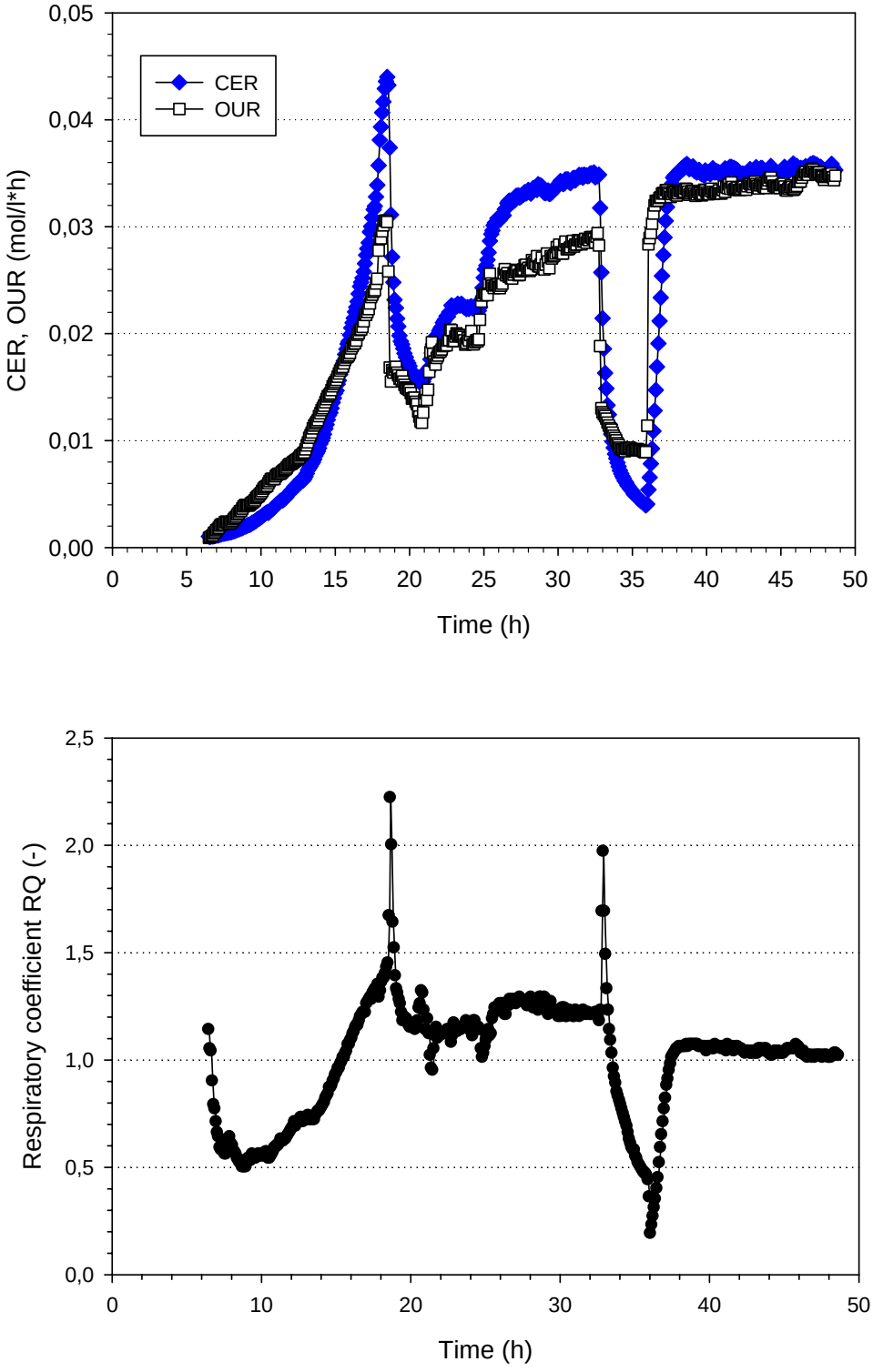


Figure 4.35: Course development of the oxygen uptake rate (OUR), the carbon dioxide evolution rate (CER) and the respiratory coefficient during the cultivation of *C. glutamicum* KY10574 in the bioreactor (3.5 L) in F1 minimal medium over 48 h at 30°C with 10 g/l glucose (t = 0); glucose feed (10 ml/h, 300 g/l, 2 • 200 ml); inoculation with 200 ml preculture (F1 medium) from shake flask cultivation; aeration rate: 0.5 L/(L • min).

The respiratory coefficient RQ (Figure 4.35) values were most of the time slightly above 1, with the exceptions of two big peaks after 18 h and 34 h. These major fluctuations can be referred to nutrient absence factors (glucose, see Figure 4.33), which are responsible for the cell metabolism acceleration or decceleration. In general, during the bioreactor cultivation more carbon dioxide was produced than oxygen was consumed. One reason for this property is the fact that the substrate glucose ($C_6H_{12}O_6$) is very rich in oxygen which can be used for metabolic reactions. Therefore, it was not necessary for the microorganisms to consume higher amounts of oxygen from the fresh air.

4.4.7.3 Bioreactor cultivation with 20 g/l glucose at t = 0 and a continuous feeding strategy for additional glucose with low pumping rates

After the first experiences with bioreactor cultivations of *Corynebacterium glutamicum* KY10574 in further experiments it was intended to enhance both the cell growth velocity and the L-methionine production. As a first step the glucose concentration in the start medium was increased to 20 g/l to improve and accelerate the cell growth of the microorganisms before starting the glucose feeding (Figure 4.36). A compromise between enhanced but controllable cell growth and a low occurrence of foam formation had to be found.

The bio dry mass course (Figure 4.36) increased much faster than in the comparable time ranges in former cultivations (see Figure 4.33). At start of the glucose feed 1, there was already a biomass concentration of 8 g/l; in the former cultivations with 10 g/l glucose initially supplied to the medium (see Figures 4.33 and 4.30) approximately 2.2 g/l were reached at this point in time. Thus, the doubling of glucose concentration right at the beginning elevated the BDM overproportionally. At the end of the cultivation process nearly 14 g/l biomass were produced. L-methionine production could also be enhanced to values of 1.15 g/l, approximately 200 mg/l more than in the former experiment (chapter 4.4.7.2). L-glycine was gained in almost the same amount of 500 mg/l after 48 h. The L-methionine production phase began with almost no delay very rapidly after the start of the cultivation. In the time period between 12 h and 30 h the L-methionine concentration increased quickly from 0.3 g/l to 1.1 g/l, afterwards the concentration remained almost constant during the last 18 h of the cultivation time.

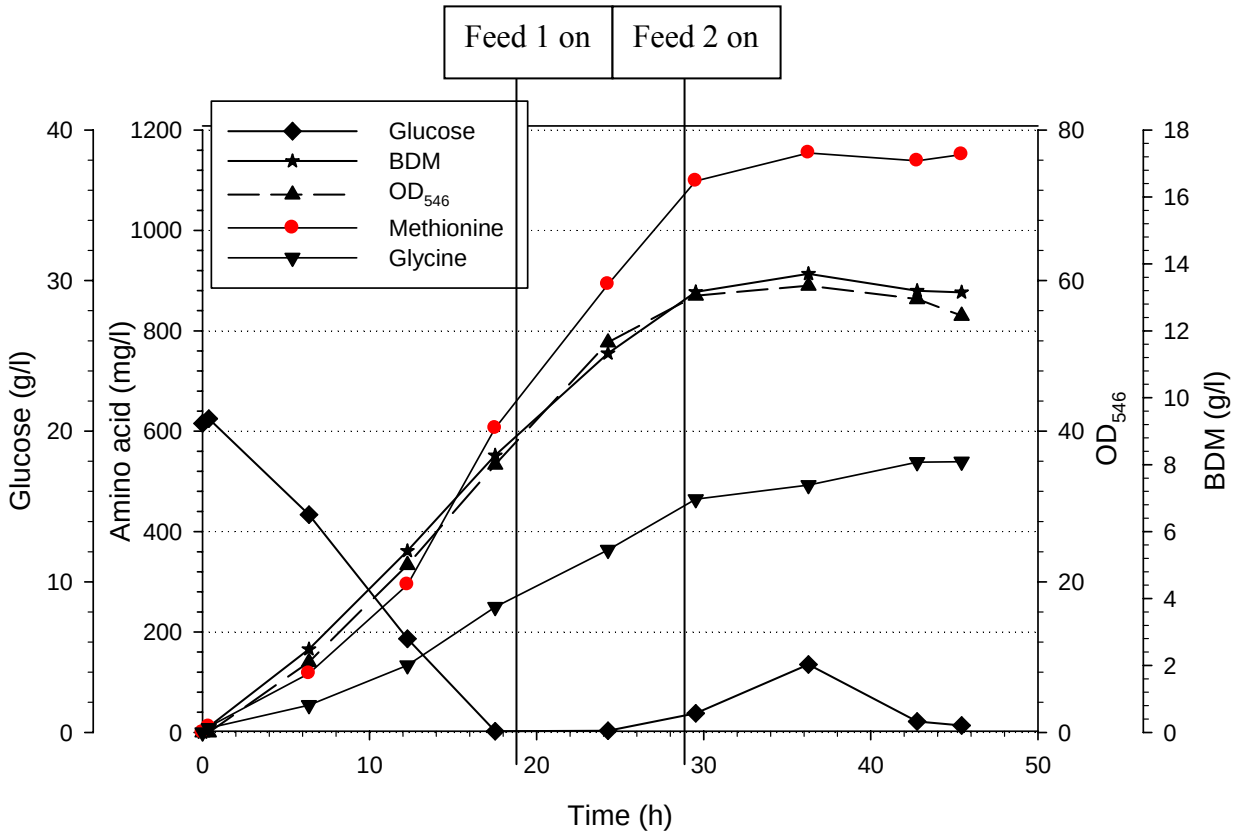


Figure 4.36: Bioreactor cultivation (3.5 L) of *C. glutamicum* KY10574 over 48 h at 30°C in F1 minimal medium with 20 g/l glucose (t =0); glucose feed with a pumping rate of 10 ml/h (2 • 200 ml, 300 g/l), presented are the courses for the development of the BDM, the glucose and the produced amino acids L-methionine and glycine; inoculation with 200 ml preculture (F1 medium) from shake flask cultivation; aeration rate: 0.5 L/(L • min); amino acid analysis with GC.

In spite of all efforts to avoid glucose accumulation a slight increase in the concentration after 36 h was observed. This amount was again consumed until the end of the process. The reason for the accumulation could be in pumping rates which might have been adjusted too highly, or in the inhibition of the cell growth through nutrient limitation. The biomass was already in the stationary phase at this time, which was an additional indicator for limitation conditions. It might be concluded that the microorganisms reorganized their metabolic pathways at that time in order to use alternative biochemical precursors and additional biochemical pathways in order to maintain a certain level of cell population. This is the current state of the art method (Eggeling et al. 1999; Gomes et al. 2005) to provide a balanced metabolism caused by nutrient shortage, at least for short-term periods.

A visual presentation of the development of the L-methionine values is shown in Figure 4.37. The development of the increased L-methionine concentration can be seen by comparing the

thickness of the spots and in relation to the 0.5 g/l L-methionine reference, but it needs to be emphasized that this method is only useful for qualitative detections.

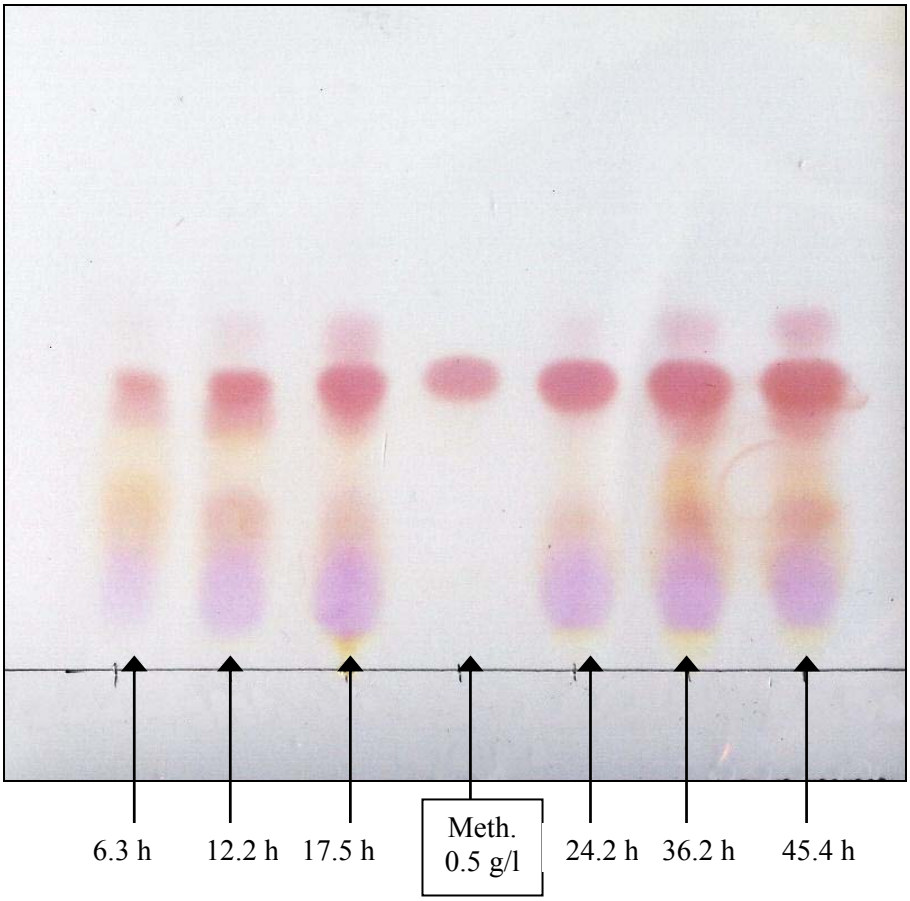


Figure 4.37: Qualitative thin layer chromatography (TLC) of the samples obtained from the bioreactor cultivation (Figure 4.36) and a reference of L-methionine (500 mg/l); detection with ninhydrin reaction.

Foam formation could not be avoided completely, particularly not during the last 18 h of the cultivation process (see Figure 4.38). But the foam did not tend to expand too much; on the contrary, there was a relatively stabilised amount of foam on top of the liquid surface. For this reason the addition of anti-foam reagent was ceased after 36 h. The foam formation in the last phase of the cultivation time was most probably caused through collapsing cells (nutrient deficiency) and the released contents from the cytosol, e.g. proteins, which can favour foaming extensively (Chmiel 2006). The pO_2 sensor system did not work correctly during this cultivation. Therefore, the stirring speed was constantly adjusted at 600 rpm (0 – 12 h) at the beginning and at 700 rpm (12 h – 48h) afterwards in order to be aware of changes in oxygen demand with increased cell density.

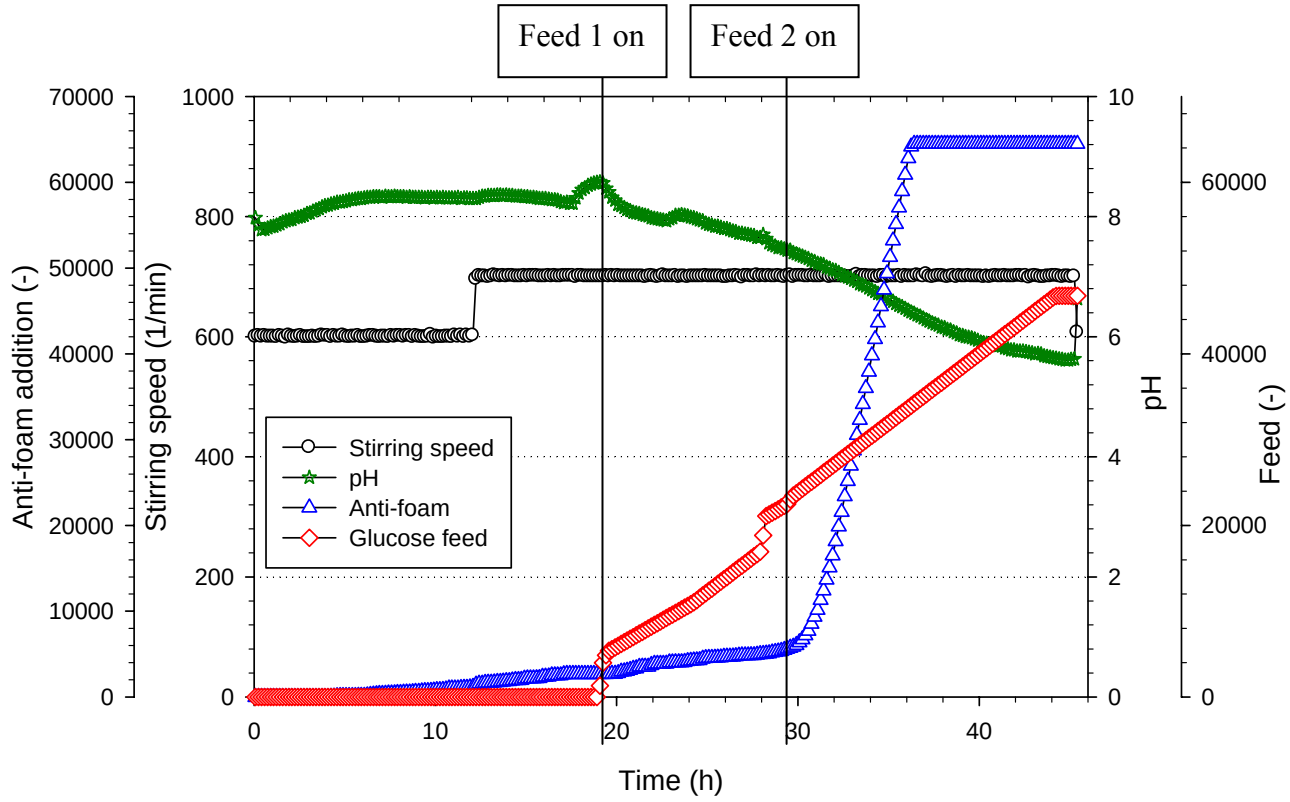


Figure 4.38: Bioreactor cultivation (3.5 L) of *C. glutamicum* KY10574 over 48 h at 30°C in F1 minimal medium with 20 g/l glucose (t = 0); presented are the courses for the development of the pH, the stirring speed and the pumping rates for the addition of the glucose feed (10 ml/h, 300 g/l, 2 • 200 ml) and the anti-foam reagent (Struktol J673); inoculation with 200 ml preculture (F1 medium) from shake flask cultivation; aeration rate: 0.5 L/(L • min).

The development of the pH is also shown in Figure 4.38. This parameter was measured online, but there was no regulation or controlling of its progression. The values ranged between 8 and 5.5 at the end of the cultivation time.

Figures 4.39 presents the exhaust gas analysis of this bioreactor cultivation. The movements in the courses for oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) mostly refer to the short term complete absence of glucose and the connected alterations in cell growth velocity which was removed quickly by starting the feeding process (Feed 1 and 2, see Figures 4.38 and 4.36). The respiratory coefficient (RQ) course development showed values around 1 with the exception of the last 10 hours of the cultivation when the values fell down under 0.5. At this point in time the demand for oxygen and consequently the formation of carbon dioxide decreased rapidly due to restricted growth conditions (glucose deficiency).

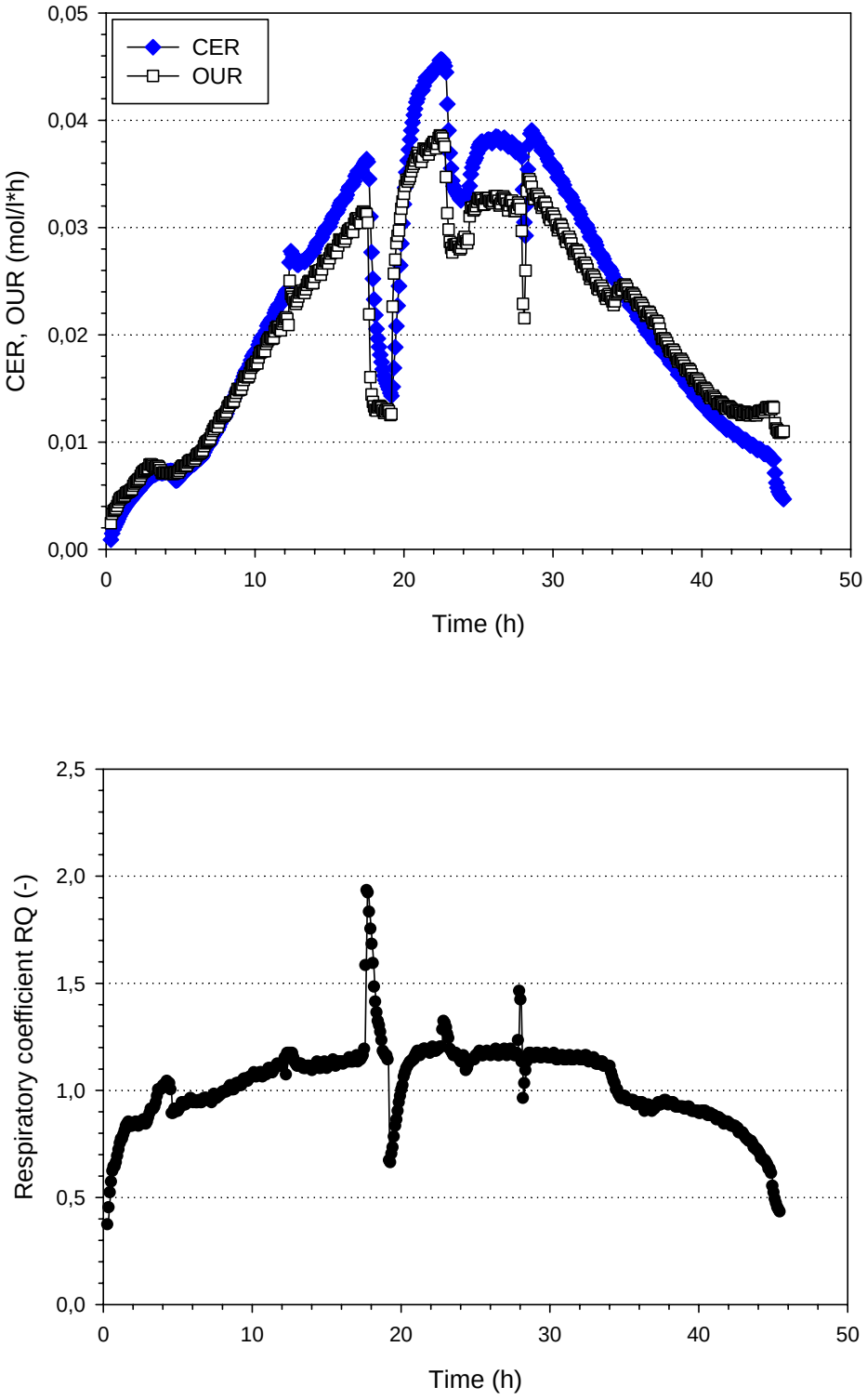


Figure 4.39: Course development of the oxygen uptake rate (OUR), the carbon dioxide evolution rate (CER) and the respiratory coefficient (RQ) during the cultivation of *C. glutamicum* KY10574 in the bioreactor (3.5 L) over 48 h at 30°C in F1 minimal medium with 20 g/l glucose (t = 0); glucose feed (10 ml/h, 300 g/l, 2 • 200 ml); inoculation with 200 ml preculture (F1 medium) from shake flask cultivation; aeration rate: 0.5 L/(L • min).

4.4.7.4 Bioreactor cultivation with 20 g/l glucose at t = 0 and a continuous feeding strategy for additional glucose with low / mid pumping rates and an effective pO_2 control system

This cultivation was performed because the pO_2 sensor did not work properly in the former experiment described in the chapter 4.4.7.3. Therefore, it was not possible to observe the impact of the automatic control between the stirring speed and the partial oxygen pressure (pO_2) on cell growth and amino acid production. The automatic control began to increase the stirring speed as soon as the pO_2 dropped under 30%. This should guarantee an optimal oxygen supply inside the bioreactor and high cell growth velocities. In former investigations (shake flask and bioreactor, see e.g. chapter 4.4.3.7) it was clearly obvious that high product concentrations of L-methionine were only available if the oxygen supply was sufficient, which was a crucial parameter in all experiments and investigations.

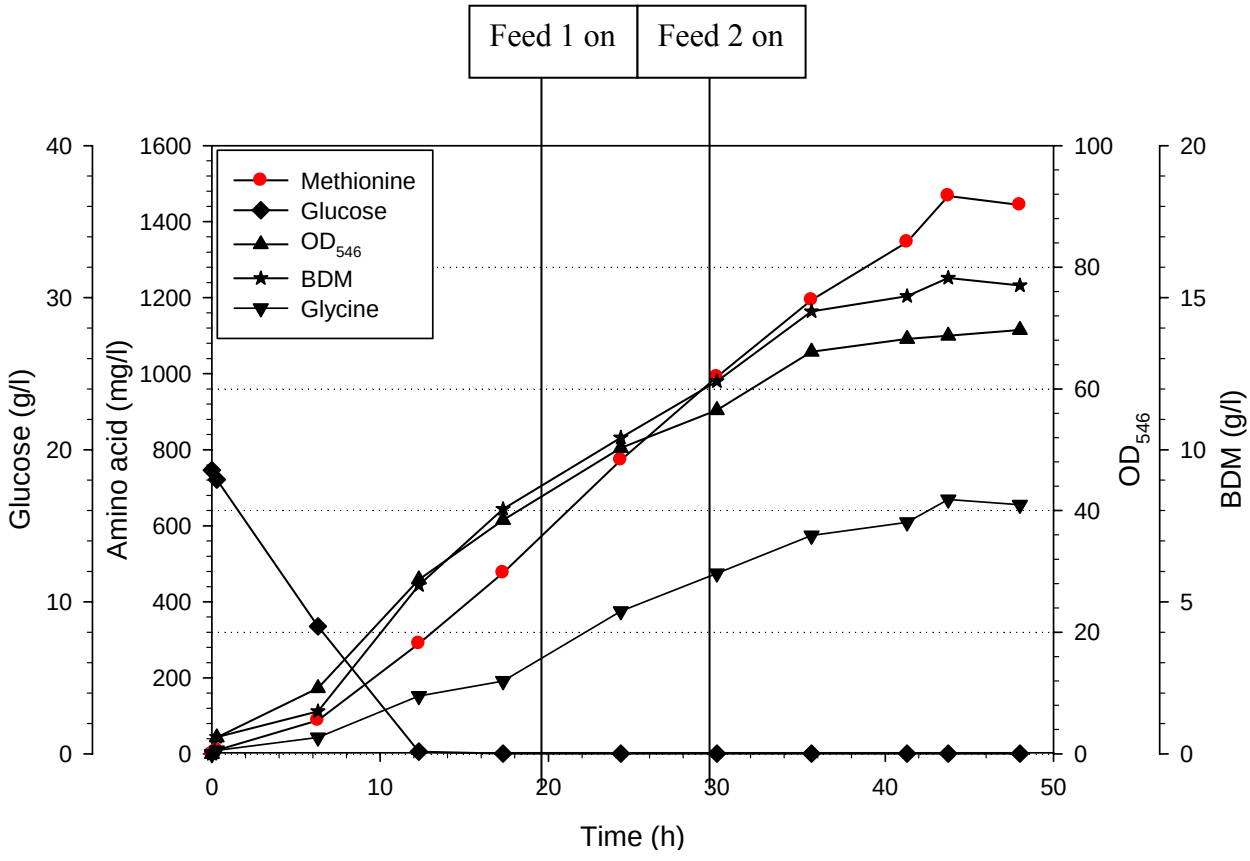


Figure 4.40: Bioreactor cultivation (3.6 L) of *C. glutamicum* KY10574 over 48 h at 30°C in F1 minimal medium with 20 g/l glucose (t = 0), glucose feed with a pumping rate of 10 - 15 ml/h (2 • 200 ml, 300 g/l), presented are the courses for the development of the BDM, the glucose and the produced amino acids L-methionine and glycine; inoculation with 200 ml preculture (F1 medium) from shake flask cultivation; aeration rate: 0.5 L/(L • min); amino acid analysis with GC.

As shown in Figure 4.40, the carbon source glucose was exhausted after 13 hours. The amount of the bio dry mass even increased to nearly 16 g/l after 48 h compared to the former cultivation with 14 g/l of bio dry mass in maximum and 20 g/l of glucose at the beginning (see Figure 4.36). The optical density (OD_{546}) reached values around 70. The L-methionine concentration values still increased when the measured values for the optical density and the BDM indicated the stationary cell growth phase. The maximum L-methionine value of 1.45 g/l was achieved after 44 h, afterwards it dropped slightly at the end of the cultivation period. A more visual and qualitative view of the development of the L-methionine concentrations during the cultivation is presented with the thin layer chromatography (TLC) in Figure 4.41. The glycine amount could be enhanced to values higher than 650 mg/l, this is more than doubled compared with the maximum in the first bioreactor cultivation (300 mg/l, Figure 4.30). It can be concluded that the glycine concentrations increase parallel to the L-methionine concentrations. Therefore, higher oxygen ratios in the cultivation broth favour also the metabolic activity of the microorganisms to produce glycine.

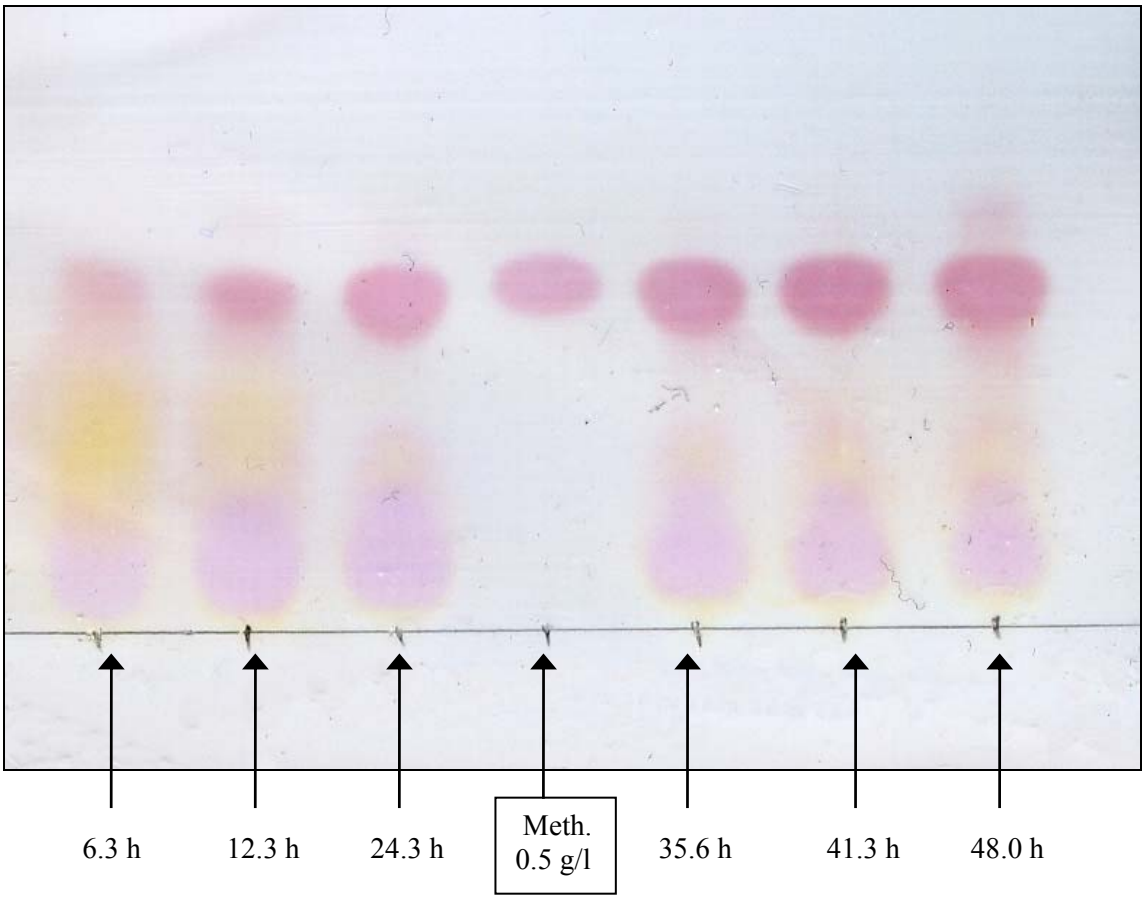


Figure 4.41: Qualitative thin layer chromatography (TLC) of the samples obtained from the bioreactor cultivation (Figure 4.40) and a reference of L-methionine (500 mg/l); detection with ninhydrin.

The major problem in this cultivation was again the foaming. Figure 4.42 shows clearly that a large amount of foam was produced during the last 12 h of the cultivation. Therefore, 200 ml anti-foam reagent was pumped into the bioreactor to reduce foam formation, which was twice the amount compared to the other bioreactor experiments. The connection between the curves for the oxygen partial pressure (pO_2) and the stirring speed is a well established fact (Chmiel 2006). The stirring speed increased immediately when the pO_2 values decreased below 30%. On the other hand the stirring speed would decrease as soon as the values for the pO_2 were above 30%. This automatic control mechanism provided optimised oxygen supply and subsequently elevated cell growth. In this case cell growth was enhanced in such a manner that the production of L-methionine was increased to values around 1.45 g/l (see Figure 4.40). But as a consequence, the high stirring speeds and the massive cell mass accumulation caused also intensive and enormous foaming. The process performance became even more difficult. Therefore, for further experiments a compromise needs to be found between high product accumulation on the one hand and lower cell growth and foaming on the other hand.

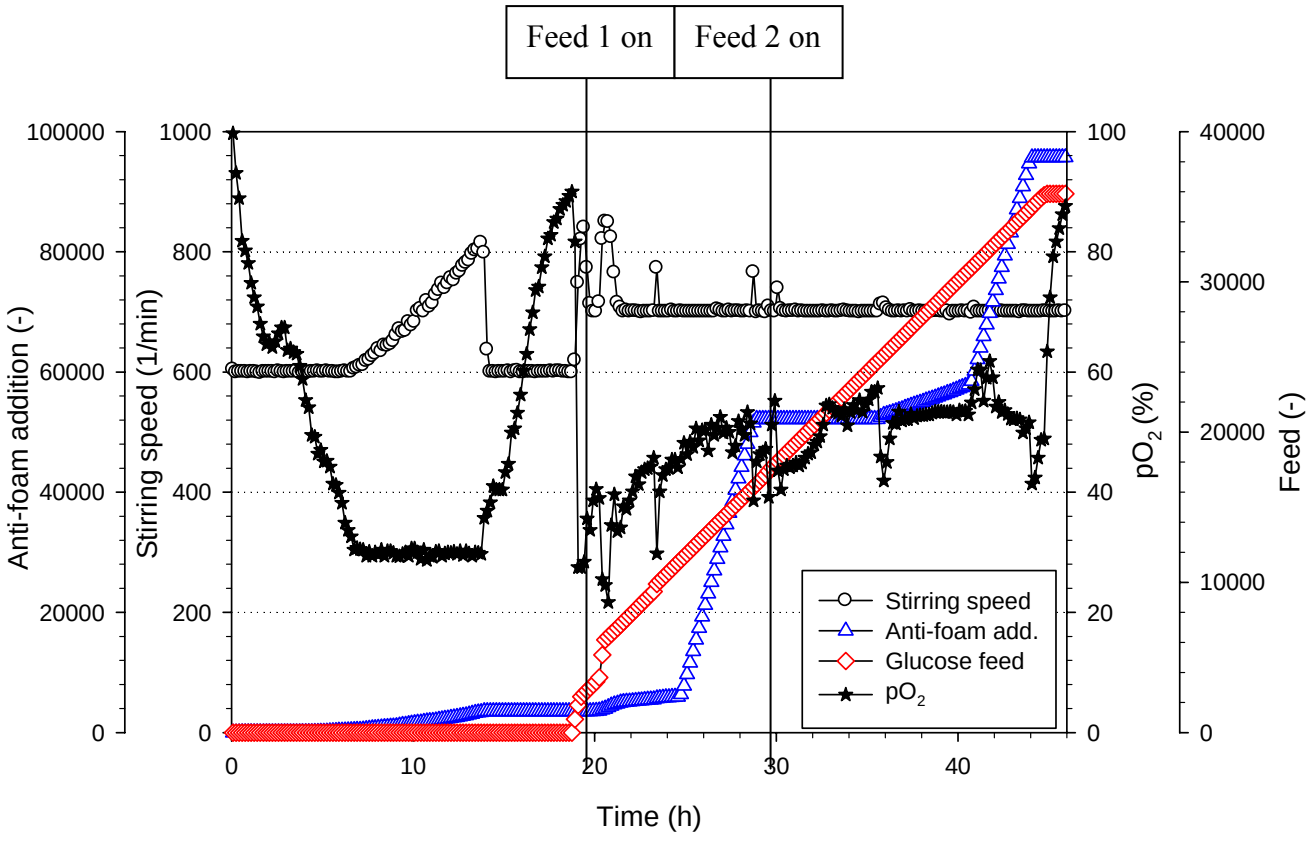


Figure 4.42: Bioreactor cultivation (3.6 L) of *C. glutamicum* KY10574 over 48 h at 30°C in F1 minimal medium with 20 g/l glucose (t = 0); presented are the courses for the development of the pO_2, the stirring speed and the pump rates for the addition of the glucose feed (10 - 15 ml/h, 300 g/l, 2 • 200 ml) and the anti-foam reagent (Struktol J673, 200 ml); inoculation with 200 ml preculture (F1 medium) from shake flask cultivation; aeration rate: 0.5 L/(L • min).

The exhaust gas analysis results are shown in Figure 4.43. There was a signal recording problem after 43 h; therefore, data for the last hours were not available. The rise of the CER and OUR courses at the beginning (maximum at 12 h) fit perfectly with the exponential biomass accumulation (see Figure 4.40). It can be concluded by comparing these data with the information shown in the Figure 4.42 + 4.40 that the metabolic activities of the microorganisms dropped significantly after 40 h. The cells began to collapse and to release a broad variety of contents from the cytosol, some of them were well known as foam causing substances (e.g. proteins). The reasons for the strong foam formation between 25 h and 30 h were most probably the high cell growth rates and the high stirring speed rates; these attendant circumstances favoured the foam formation. A method to inhibit the extensive foaming and to provide even improved conditions for oxygen supply might be the gassing of the cultivation broth with pure oxygen, although handling with pure oxygen is not easy. A further possibility to avoid foaming is the application of different chemical anti-foam reagents like e.g. sun flower oil or rapeseed oil (colza oil).

The development of the respiratory coefficient (RQ) in Figure 4.43 was characteristic for all cultivations in the bioreactor with a course development around 1. Although the oxygen supply conditions were improved for this cultivation compared to the last one, these alterations caused simultaneously increased cell growth and even higher oxygen demands. The improvement of the oxygen supply increased automatically the cell mass formation and subsequently led to even higher demands for oxygen. The production of carbon dioxide during the cultivation of *C.glutamicum* KY10574 cells was higher than the uptake rate of oxygen from fresh air due to the fact, that the microorganisms are able to use the oxygen parts of the substrate glucose ($C_6H_{12}O_6$) for metabolism activities.

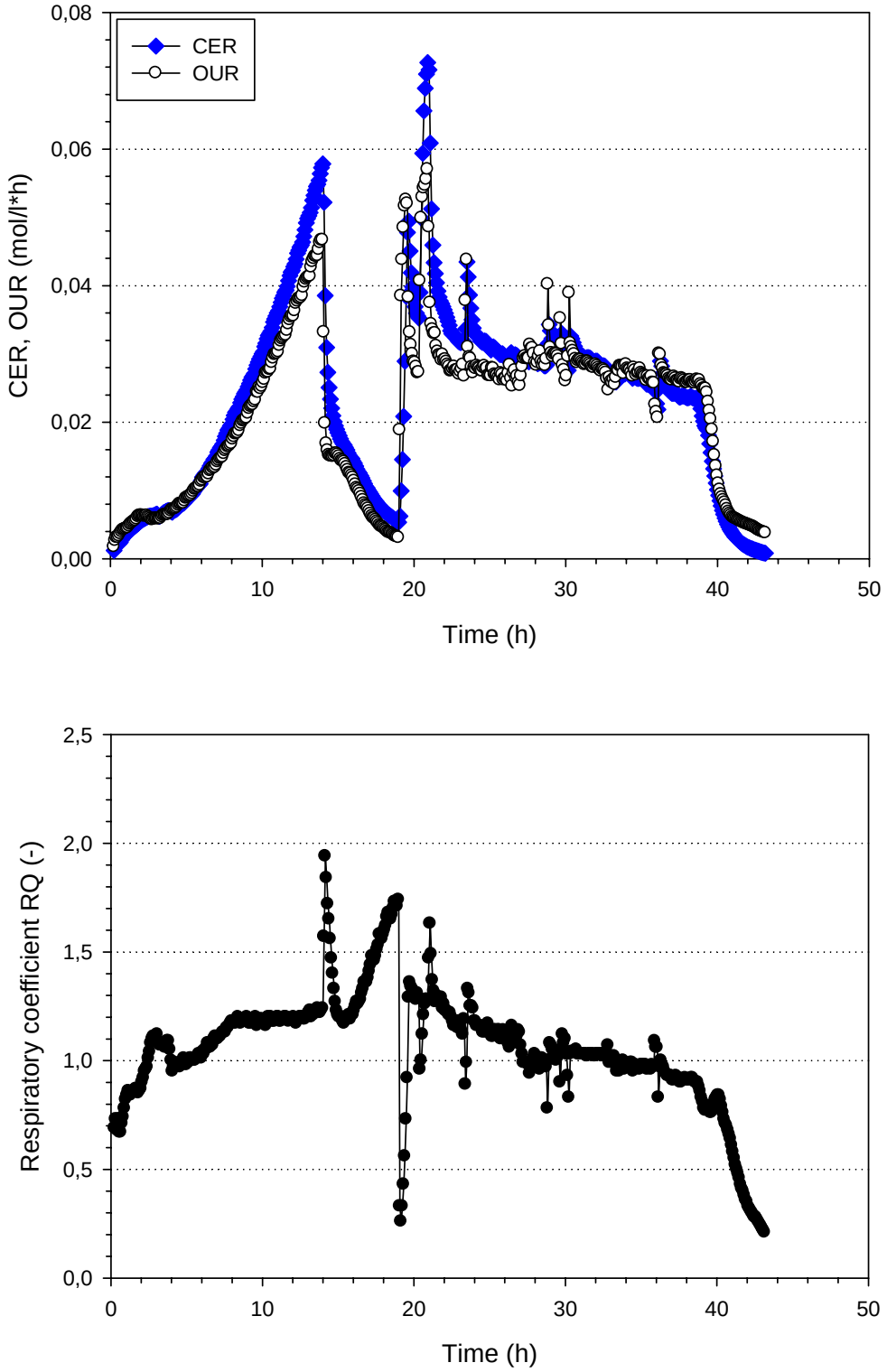


Figure 4.43: Course development of the oxygen uptake rate (OUR), the carbon dioxide evolution rate (CER) and the respiratory coefficient (RQ) during the cultivation of *C. glutamicum* KY10574 in the bioreactor (3.6 L) in F1 minimal medium over 48 h at 30°C with 20 g/l glucose (t = 0); glucose feed (10 - 15 ml/h, 300 g/l, 2 • 200 ml); inoculation with 200 ml preculture (F1 medium) from shake flask cultivation; aeration rate: 0.5 L/(L • min).

4.4.7.5 Summary about the bioreactor cultivations with *Corynebacterium glutamicum* KY10574

Considering the results from the bioreactor experiments the strategy to perform the cultivations with a low initially supplied glucose concentration and a following continuous feeding strategy was successful. Additionally, this was absolutely necessary in order to avoid massive foam formation. It was important to get control over cell growth velocity and foam formation using a limitation factor as glucose to be able to cultivate the microbial strain under best conditions for amino acid production and process handling. Anaerobic conditions in the cultivation broth resulted directly in a significantly diminished production of L-methionine (see Figure 4.30).

It was advantageous to double the initially supplied sugar concentration from 10 to 20 g/l in order to enhance the cell growth velocity and subsequently the L-methionine production in the first hours of the cultivation until the glucose was consumed. Usually, the foam formation appeared in phases of nutrient deficiency (glucose) and under anaerobic conditions. The anaerobic conditions occurred when the cell growth was too fast (no effective limitation through the sugar concentration) and the consumption of oxygen was too strong (see Figure 4.31). Proteins and other foam developers were released after the cell collapse. Therefore, the highest L-methionine concentrations [1.15 g/l (Figure 4.36) and 1.46 g/l (Figure 4.40)] could be achieved using 20 g/l of initially supplied sugar and an effective mechanism for oxygen transport into the liquid cultivation broth by automatic control between the partial oxygen pressure and the stirring speed (see Figure 4.42).

Table 4.2 shows the development of significant parameters in comparison of all bioreactor cultivations. Apart from the total amount of glucose, the used pumping rates for the glucose feed and the maximum reached values for the bio dry mass and L-methionine concentrations, there is an overview about the development of the yield coefficients ($Y_{X/S}$, $Y_{P/S}$, $Y_{P/X}$). The values for these coefficients are applied to the data derived at the end of the cultivations. It can be seen in Table 4.2 that the value for the yield coefficient $Y_{X/S}$ from bioreactor cultivation 1 (chapter 4.4.7.1) with 0.37 was the highest one. The conversion from substrate to biomass was most effective in this cultivation; the maximum bio dry mass value in this cultivation (16.2 g/l, see Table 4.2) was also the highest compared to the other cultivations. The values for the yield coefficient $Y_{X/S}$ drop to a minimum of 0.21 (bioreactor cultivation 3) and rise again to 0.3 at the end of bioreactor cultivation 4.

On the other side the values for the yield coefficient $Y_{P/X}$ show an increase from 0.025 (first cultivation) to 0.094 in the fourth cultivation. The ratio of product (P, L-methionine) to bio dry mass (X) is much more advantageous in the cultivations with an effective limitation of the cell growth through glucose limitation. The same trend is visible for the yield coefficient $Y_{P/S}$; the values for this coefficient increased continuously from the first (0.095) to the fourth cultivation (0.028). So the product oriented yield coefficients ($Y_{P/S}$, $Y_{P/X}$) emphasize the importance of a continuous fed batch cultivation process.

Therefore, the yield coefficients reveal that the first bioreactor cultivation was effective in fast biomass accumulation but very non-effective in the L-methionine production which was the crucial factor in all experiments. On the contrary, it is shown that too high cell growth velocities which caused anaerobic conditions in the cultivation broth are extremely inapprobriate for the L-amino acid production (see the course development for the oxygen partial pressure pO_2 in Figure 4.31 and corresponding values for L-methionine production in Figure 4.30).

Table 4.2: Comparison of the 4 bioreactor cultivations (BR) which are described in the chapter 4.4.7 on the basis of significant biochemical engineering parameters, presented in chronological order from chapter 4.4.7.1 – 4.4.7.4 (BR 1-4); the values for yield coefficients ($Y_{X/S}$ / $Y_{P/S}$ / $Y_{P/X}$) are applied to the data obtained at the end of the cultivations.

Bioreactor (BR) and initially supplied glucose	**BR 1: 10 g/l**	**BR 2: 10 g/l**	**BR 3: 20 g/l**	**BR 4: 20 g/l**
Pumping rate mode for glucose feed (ml/h)	70 ml/h (11-14 h); 15 ml/h	10 ml/h	10 ml/h	10 – 15 ml/h
Glucose total (g)	150	150	180	180
BDM_{max} (g/l)	16.2	11.5	12.9	15.6
L-$Methionine_{max}$ (mg/l)	442	934	1,154	1,467
$Y_{X/S}$ (g bio dry mass / g glucose)	0.37	0.27	0.21	0.30
$Y_{P/X}$ (g L-methionine / g bio dry mass)	0.025	0.081	0.092	0.094
$Y_{P/S}$ (g L-methionine / g glucose)	0.010	0.021	0.022	0.028
$Glycine_{max}$ (mg/l)	305	511	539	669

Table 4.2 shows also the development of the glycine concentrations. It is remarkable that glycine is, apart from L-methionine, the only amino acid which was produced in the bioreactor cultivations in noticeable amounts. Glycine was also a product in the shake flask cultivations (see e.g. Figures 4.19 -4.24). The glycine concentrations also increased considerably and in parallel to the increasing L-methionine concentrations (compare Figures 4.30, 4.33, 4.36, 4.40) but were always clearly below the L-methionine concentrations. In the first bioreactor cultivation (chapter 4.4.7.1) with partially anaerobic conditions and unsufficient oxygen supply the glycine values were around 300 mg/l in maximum, in the last cultivation with improved oxygen conditions the maximum value was 669 mg/l; so the concentration was more than doubled. Therefore, it can be concluded that the glycine production is favoured in the same way as L-methionine production by improved oxygen supply and oxygen transport into the cultivation broth.

The amino acids L-alanine, L-valine and in some cases L-glutamic acid which were produced in noticeable amounts in the shake flask cultures (see Figures 4.19 – 4.24) disappear in the bioreactor cultivations. The better conditions for oxygen supply in the bioreactor scale favour the production of L-methionine and glycine; the production of other amino acid by-products stopped completely.

In the case of L-alanine, which was in some cases the dominant produced amino acid in the shake flasks (Figures 4.22 + 4.23), there is another explanation. There was no production due to the glucose limited conditions in the bioreactor cultivations. As shown before in the shake flask cultivations (Figures 4.19 – 4.24) the L-alanine concentrations dropped rapidly after the glucose was consumed or occurred only in low concentrations in the media.

The bioreactor experiments which are described in this thesis are fed batch cultivations (closed systems). In following experiments in the bioreactor scale it would be interesting to observe the influence of a continuous cultivation process in bioreactor with a continuous feeding of nutrient solutions and a continuous draining off (open system) on the L-methionine accumulation in the cultivation broth. This process will be performed usually in continuous stirred tank reactor (CSTR) systems.

4.4.8 Investigation of total L-methionine concentrations (extra- and intracellular) of the strain *C. glutamicum* KY10574 compared to the strain *C. glutamicum* DSM20300

C. glutamicum is able to enrich distinct amounts of amino acids inside the cells, as has been investigated e.g. for glutamic acid production (Clement et al. 1986). Several experiments were carried out to improve the release of amino acids from the intracellular volume to the extracellular milieu, e.g. by modification of the cell wall density through biotine limitation during cultivation (Leuchtenberger 1996). The release of the amino acid molecules is important in order to prevent the deactivation of amino acid production because the cells are constrained to end this process in order to protect their stability which is at risk through higher enrichments of amino acids in the intracellular milieu.

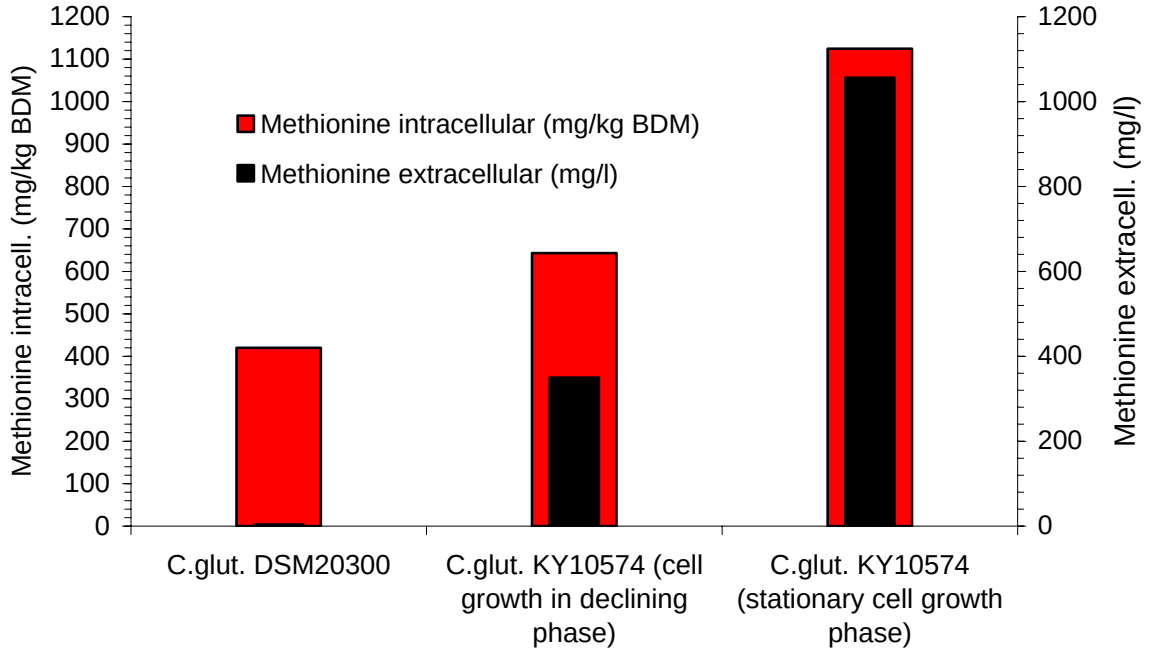


Figure 4.44: Comparison of *C. glutamicum* DSM20300 and KY10574 intracellular amounts of L-methionine, measured after cell disruption with an ultrasonic homogenizer, the extracellular amounts are shown for comparison.

The measured amounts of L-methionine (Figure 4.44) after cell disruption were for both strains extremely minimal, values around 420 mg/kg bio dry mass (*C. glutamicum* DSM20300) and 643.9 / 1124.9 mg/kg bio dry mass (*C. glutamicum* KY10574) could not be

considered as an intracellular enrichment of L-methionine. The strain *C. glutamicum* DSM20300 which was not able to produce L-methionine in remarkable amounts (in this case 3.7 mg/l) showed almost the same intracellular amount of L-methionine as the strain *C. glutamicum* KY10574. It is normal for the strain *C. glutamicum* KY10574 to exhibit more L-methionine intracellularly in the stationary phase than in the decline phase, because in this phase the metabolism is still partially working and producing L-methionine which has to be found naturally in the cytosol before releasing it to the outside. Under these circumstances even higher amounts would have been expected by measuring of L-methionine in the exponentiell production phase of the strain. It was even astonishing that the results for L-methionine concentrations in the cytosol were that low compared to the high amounts of L-methionine measured in the supernatant (see Figure 4.44). The results obtained from these experiments clearly point out that the strain *Corynebacterium glutamicum* KY10574 has no problems releasing its amino acids in the extracellular volume by cultivating in F1 medium.

4.4.8.1 Protein hydrolysis of *C. glutamicum* KY10574 bio dry mass

The complete protein hydrolysis of *C. glutamicum* KY10574 was performed to get a result about the complete L-methionine content obtained in the bioreactor cultivation which is described in chapter 4.4.7.3. At the end of the cultivation 12.45 g/l bio dry mass was produced; the concentration of L-methionine in the supernatant was 1151.15 mg/l (see Figure 4.36). The bio mass was separated from the supernatant and freeze dried (after a washing procedure). After total hydrolysis of the biomass including proteinsa determination of the amino acid degree of methionine, threonine and lysine in the bio dry mas was performed. These amino acids are the most important additives in animal feed nutrition. The hydrolysis and the amino acid determination was carried out at the LUFA (Landwirtschaftliche Untersuchungs- und Forschungsanstalt) in Speyer. The results are shown in Table 4.3.

Table 4.3: Amino acid content of the bio dry mass from *C. glutamicum* KY10574 after protein hydrolysis, obtained at the end of the bioreactor cultivation (chapter 4.4.7.3).

Methionine (%)	Lysine (%)	Threonine (%)
1,47	4,33	3,52

The results shown in Table 4.3 had to be converted in order to combine them with the methionine amount in the supernatant. At the end of the bioreactor cultivation 12.45 g/l bio dry mass (BDM) were gained. After connecting these values with the rule of proportion method, the results of amino acids obtained after protein hydrolysis can be specified as concentrations, as shown in Figure 4.45. It was pointed out clearly, that in all shake flask and bioreactor experiments with this strain the major amount of methionine is referred to the supernatant but for lysine and threonine amounts the major source was the bio mass content.

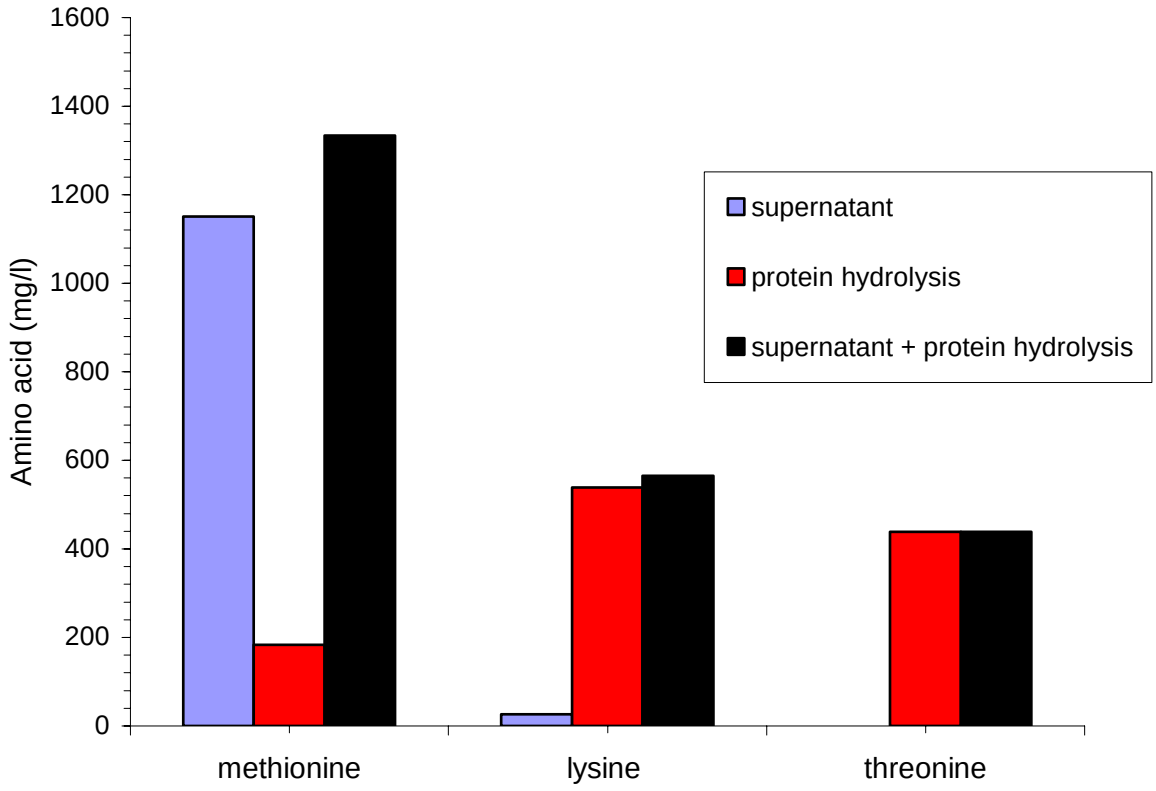


Figure 4.45: Complete contents of the amino acids methionine, lysine and threonine obtained in bioreactor cultivation (chapter 4.4.7.3, see Figure 4.36); presented are the concentrations in the supernatant, the bio dry mass (BDM) and both summed up.

As for L-lysine, for the industrial application the complete cultivation broth was dried (e.g. spray drying) and mixed to animal feed in suitable ratios (see Figure 2.8). This procedure avoids the additional costs for down stream processing and makes the use of this biotechnically gained amino acid as feed additive more competitive against the chemical process (Leuchtenberger 1996). It has been recommended to supply laying hens with animal feed containing approximately 0.3% DL-methionine and 0.61% L-lysine (Keshavarz 2003; Noftsger et al. 2003; Misciatelli et al. 2003). The supplementation with lysine, methionine and threonine is necessary because the established sources for animal feed (wheat, soy bean) exhibit deficiencies of these amino acids compared to the nutritional requirements of poultry breeding (Pack 2004).

5 Summary / Outlook

The first objective at the beginning of this research was the screening for L-methionine overproducers using *Corynebacterium glutamicum* DSM20300. This strain has the capability to produce L-glutamic acid and was the starting point for numerous studies on amino acid production. The method to generate the overproducing mutants was the random mutagenesis by ultraviolet radiation and following selection with methionine analogues / anti-metabolites, e.g. ethionine or norleucine. It was possible to create mutants and to measure production capabilities under high throughput conditions. The selection system turned out to be the crucial problem. The application of anti-metabolites was a necessary, but not a sufficient criterion. The microorganisms which probably owned the abilities to overproduce L-methionine had to grow on media with anti-metabolite contents. It was not imperative, however, that these mutants gained the properties to produce L-methionine. In spite of all experimental efforts, an overproducer of L-methionine could not be obtained by this method.

The strain *Corynebacterium glutamicum* ATCC21608 was bought from the ATCC because indications on L-methionine production abilities had been reported (Nakayama et al. 1973). After several tests in shake flask cultivations with different media it could be proved that the delivered strain material was able to overproduce L-alanine (ca. 200 mg/l), but not L-methionine. Perhaps the strain material was too old and inactive after a long period of storage at the ATCC.

The Japanese company Kyowa Hakko Kogyo Ltd. kindly provided the strain *Corynebacterium glutamicum* KY10574. This strain was able to produce L-methionine.

In the first experiments with this strain in the shake flask scale in a combination of a complex preculture and a minimal main medium a broad variety of amino acids (e.g. lysine, glycine, alanine, methionine) could be observed. Lysine was the dominant amino acid (nearly 700 mg/l) under those conditions, methionine was produced in amounts of ca. 125 mg/l. The production of lysine began in the phase of precultivation in the complex medium, therefore, the product spectrum was preadjusted to lysine through the usage of complex ingredients.

The use of a minimal medium which was able to deliver all nutrients for the cell growth of *Corynebacterium glutamicum* KY10574 provided a way to alter the product spectrum, so that a complex precultivation was not necessary any more. It was possible to establish a minimal medium which guaranteed best cell growth conditions. The product spectrum was changed completely, there was no remarkable production of lysine any more. L-methionine was now the dominant amino acid, followed by glycine. It could be shown in several shake flask experiments with this medium that high shaking rates and a distinct concentration of glucose (around 55 g/l) could improve the production capability of L-methionine considerably. Sufficient oxygen supply was important for rising the L-methionine production with *C. glutamicum*, and values of 1.45 g/l L-methionine could be achieved using a preculture. The use of higher glucose concentrations than 70 g/l favoured the production of another amino acid, L-alanine.

In the bioreactor scale, it was necessary to cultivate *Corynebacterium glutamicum* KY10574 with a limitation factor in order to control cell growth velocity and to guarantee sufficient oxygen supply for optimised L-methionine production. The glucose concentration was used in the cultivations as limitation factor, the balance between high cell growth and L-methionine production on the one hand and controlled growth with sufficient oxygen rates on the other hand could be improved crucially. The production of L-methionine in the bioreactor scale could be enhanced from 425 mg/l in the first cultivation to 1.4 g/l in the last cultivation. A major problem was foam formation in the bioreactor due to uncontrolled growth with intensive cell mass accumulation and the cell collapse under anaerobic and nutrient limited conditions at the end of the cultivations. This problem could be solved mostly by an automatic foam sensor system connected with an anti-foam reagent feed. But still high amounts of this reagent were necessary to prevent foaming in an adequate way.

The major differences in amino acid production after media alteration emphasized the importance of the medium components for L-methionine production. After extensive investigations on the influence of the carbon source (glucose) on the L-methionine production, the improvement and optimisation of the nutrients providing ammonia (NH_4^+), sulphate (SO_4^{2-}) and phosphate (PO_4^{3-}) could also help to enhance production. With L-methionine overproducing strain *C. glutamicum* KY10574 as starting point even the experiments in random mutagenesis might be more effective in order to achieve further improvements in production.

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7 Abbreviations

ACQ	6-amino-quinolyl-N-hydroxy-succinimidyl-carbamate
ATCC	American Type Culture Collection
BDM	Bio dry mass
BY	Bouillon yeast extract
BSA	Bovine serum albumin
CER	Carbon dioxide evolution rate
DSM	German Collection of Microorganisms
EMS	Ethyl-methanesulfonate
GC	Gas chromatography
HPLC	High performance / pressure liquid chromatography
IMH	Indolyl-methyl-hydantoin
kDa	Kilodalton
MOPS	Morpholine propane sulphonic acid
MS	Mass spectometry
MTEH	Methyl-thio-ethyl-hydantoin
nm	Nanometer (wavelength)
n	Stirring speed
NTG	N-methyl-N'-nitro-nitrosoguanidine
OD	Optical density
OPA	Ortho-phtaldialdehyde
OUR	Oxygen uptake rate
pO_2	Oxygen partial pressure
PITC	Phenylisothiocyanate
q_{CO2}	= CER (based on molar mass)
q_{O2}	= OUR (based on molar mass)
RQ	Respiratory coefficient
THF	Tetra hydro folate
TLC	Thin layer chromatography
TOF	Time of Flight
$Y_{X/S}$	Yield coefficient [(g) bio dry mass / (g) substrate]
$Y_{P/S}$	Yield coefficient [(g) product / (g) substrate]
$Y_{P/X}$	Yield coefficient [(g) product / (g) bio dry mass]
UV	Ultraviolet light

8 Attachment

Table A.1: Additional amino acids in minor concentrations measured in the cultivation with KH1 + KH2 medium (see Figures 4.09 + 4.10; chapter 4.4.1).

KY10574				
Time (h)	L-leucine (mg/l)	L-iso-leucine (mg/l)	L-valine (mg/l)	L-glutamic acid (mg/l)
0	42.3	17.7	21.1	6.3
24	46.7	20	27.04	15.8
48	40.8	16.8	30.1	28.8
72	16.8	5.1	40	29.4
96	19.6	7.9	37.8	35.3
120	66.1	30.7	68.03	64.5
144	74.29	39.6	76.2	65.9
168	69.3	36.6	79.3	65.27
240	9.3	5.2	28.4	18.1

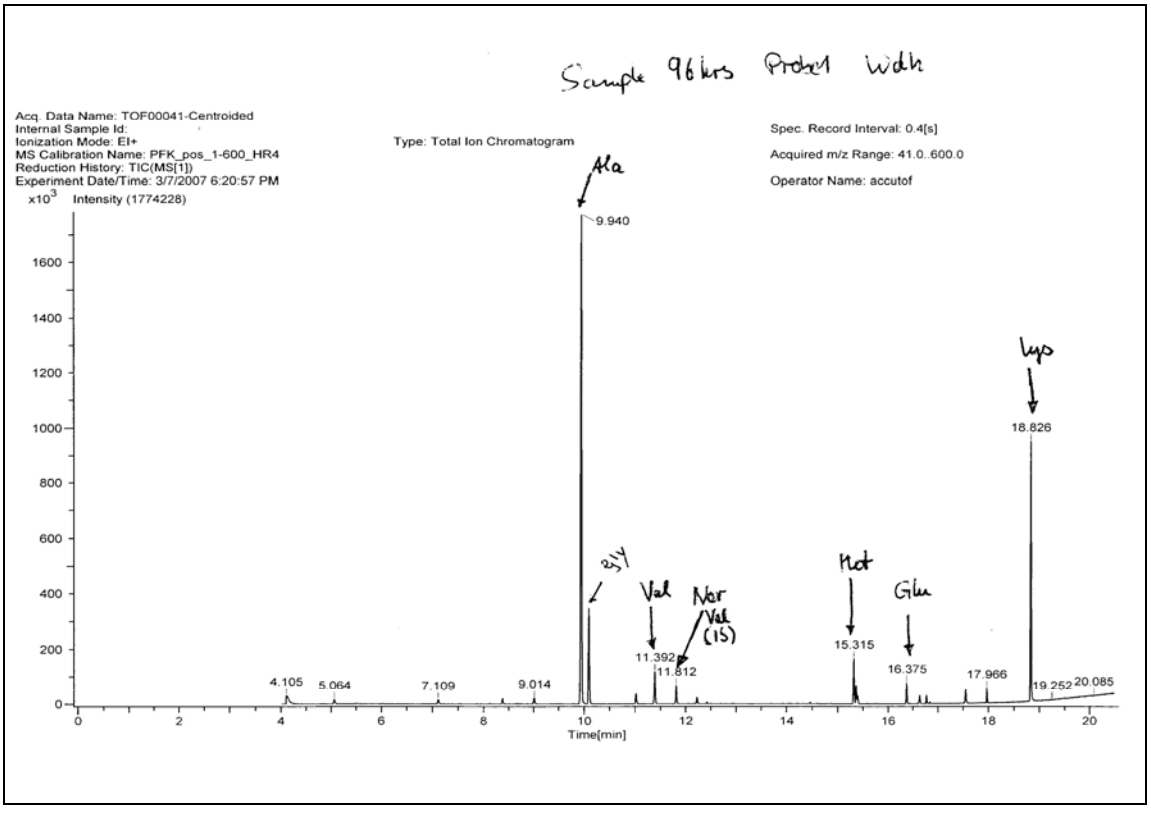

Figure A.1: Diagram of the GC measurement (performed at the Institute of Organic Chemistry, TU Braunschweig) and the amino acid identification of the sample after 96 h cultivation time from the cultivation with KH2 medium showed in the results in the Figure 4.10 (chapter 4.4.1).

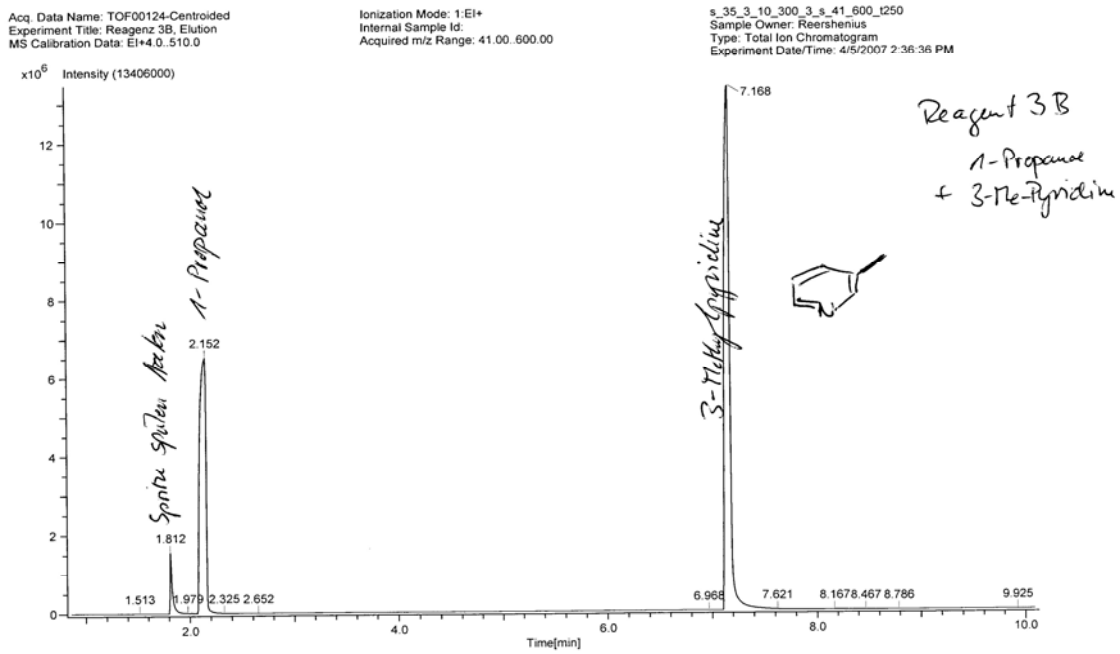

Figure A.2: Diagram of the GC measurement (performed at the Institute of Organic Chemistry, TU Braunschweig) of the reagent **3B** from the derivatization kit for amino acids (Phenomenex company) to identify the important substances for the modification of the amino acids, in this case the catalysator 3-methyl pyridine (see chemical reaction in chapter 4.4.1.1 and Table 3.10).

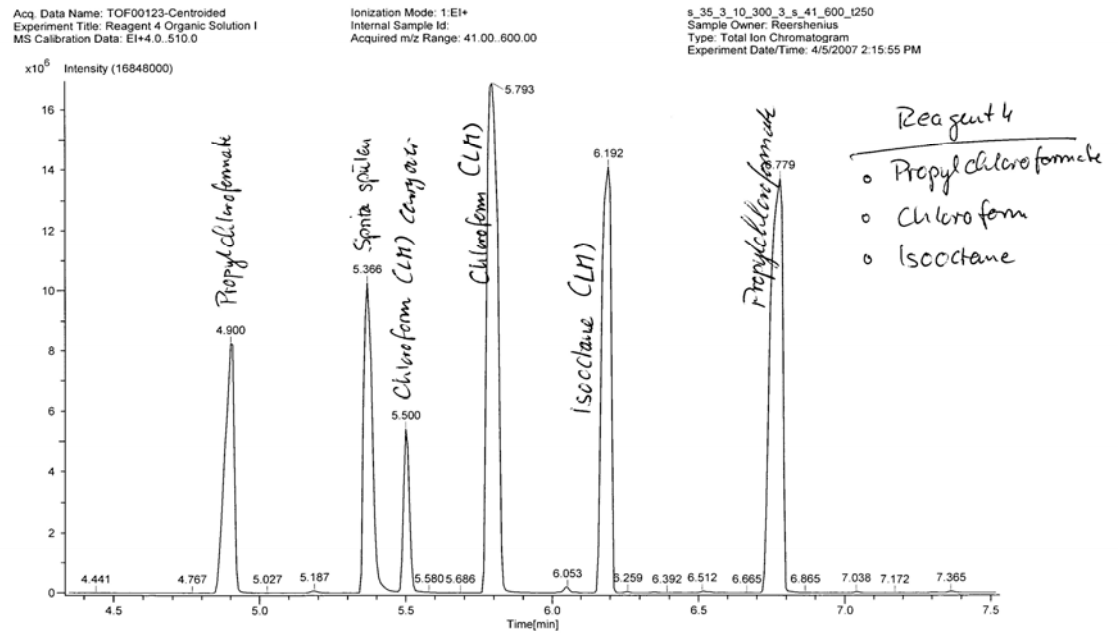

Figure A.2: Diagram of the GC measurement (performed at the Organic Chemistry, TU Braunschweig) of the reagent **4B** from the derivatization kit for free physiological amino acids (Phenomenex company) to identify the important substances for the modification of the amino acids, in this case the propyl chloro formate substances (see chemical reaction in chapter 4.4.1.1 and Table 3.10).

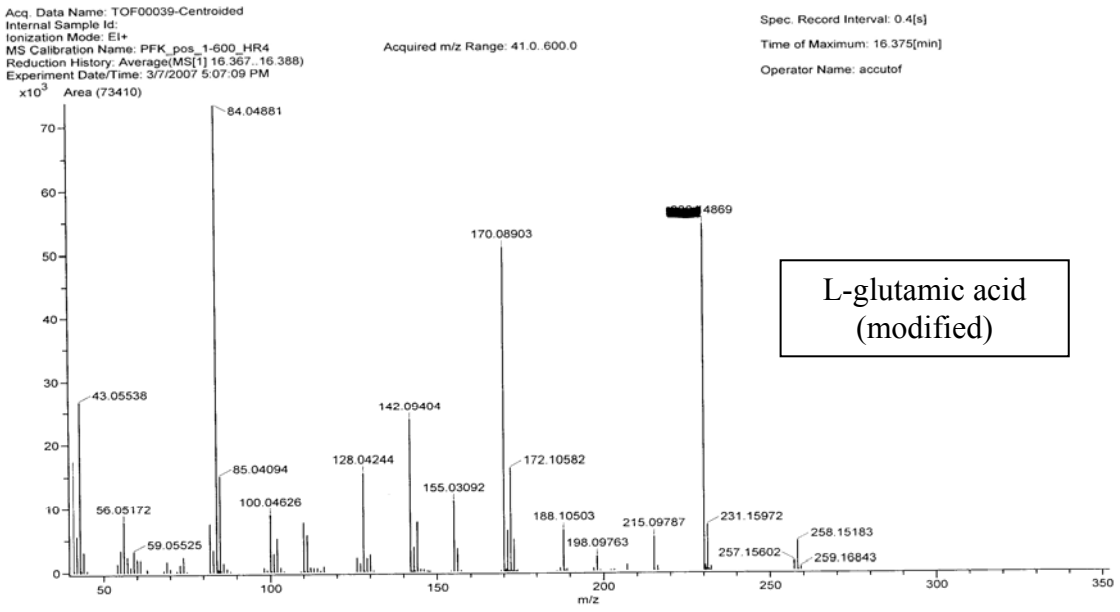

Figure A.3: Diagram of the mass spectrometry fragments (MS; performed at the Organic Chemistry, TU Braunschweig) of **modified L-glutamic acid (see Figure 4.15)** obtained through GC-MS from the sample taken after 120 h cultivation time (for the cultivation see Figure 4.10 / chapter 4.4.1).

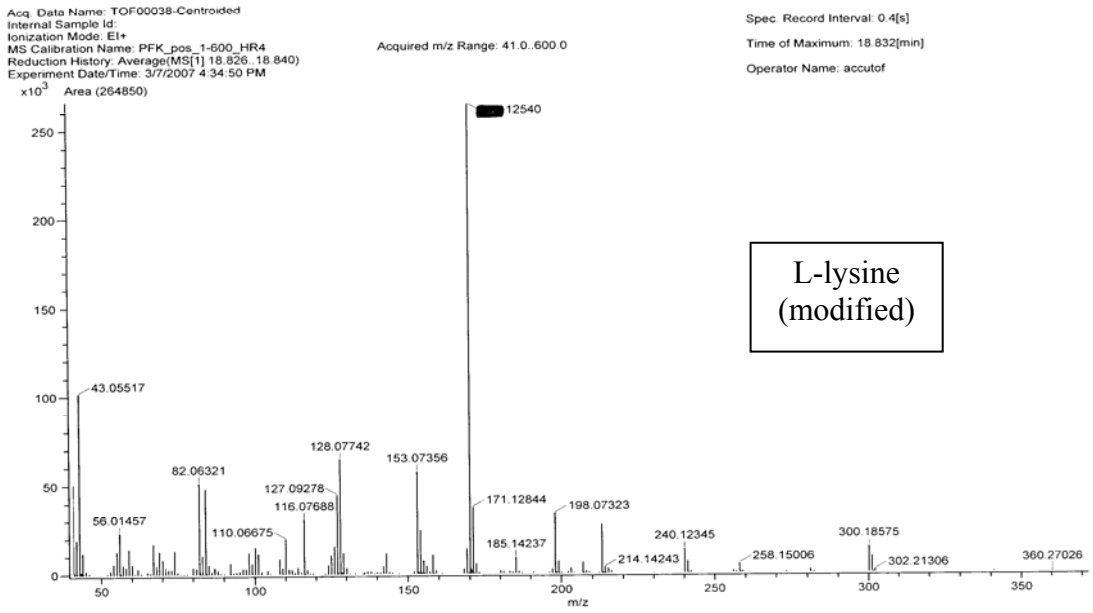

Figure A.4: Diagram of the mass spectrometry fragments (MS; performed at the Organic Chemistry, TU Braunschweig) of **modified L-lysine (see Figure 4.15)** obtained through GC-MS from the sample taken after 120 h cultivation time (for the cultivation see Figure 4.10 / chapter 4.4.1).

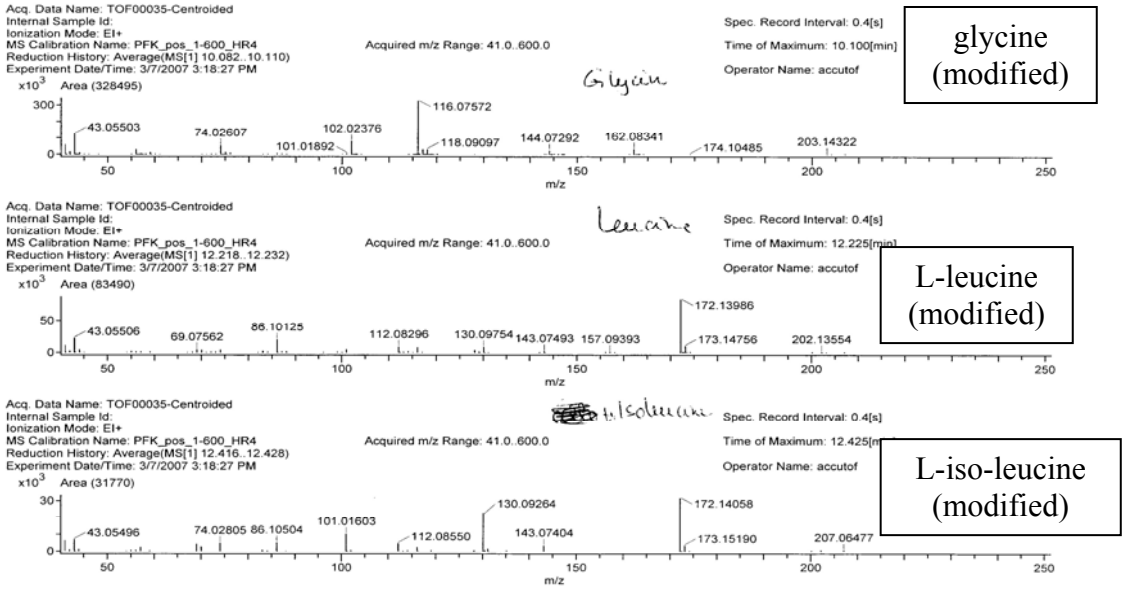

Figure A.5: Diagram of the mass spectrometry fragments (MS; performed at the Organic Chemistry, TU Braunschweig) of **modified glycine**, **modified L-leucine** and **modified L-iso-leucine (see Figure 4.15)** obtained through GC-MS from the sample taken after 120 h cultivation time (for the cultivation see Figure 4.10 / chapter 4.4.1).

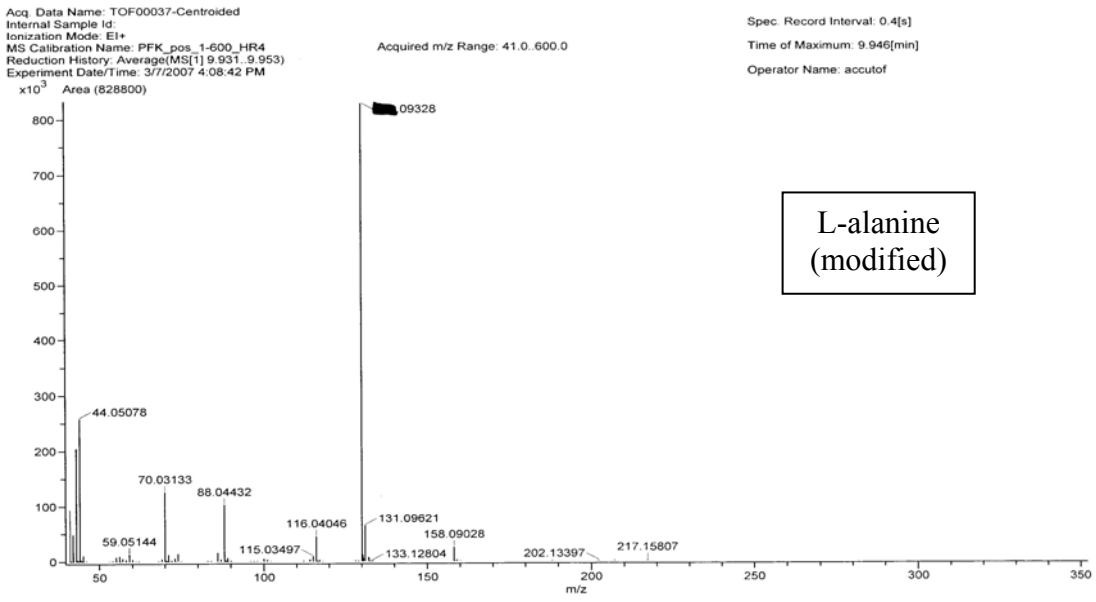

Figure A.6: Diagram of the mass spectrometry fragments (MS; performed at the Organic Chemistry, TU Braunschweig) of **modified L-alanine (see Figure 4.15)** obtained through GC-MS from the sample taken after 120 h cultivation time (for the cultivation see Figure 4.10 / chapter 4.4.1).

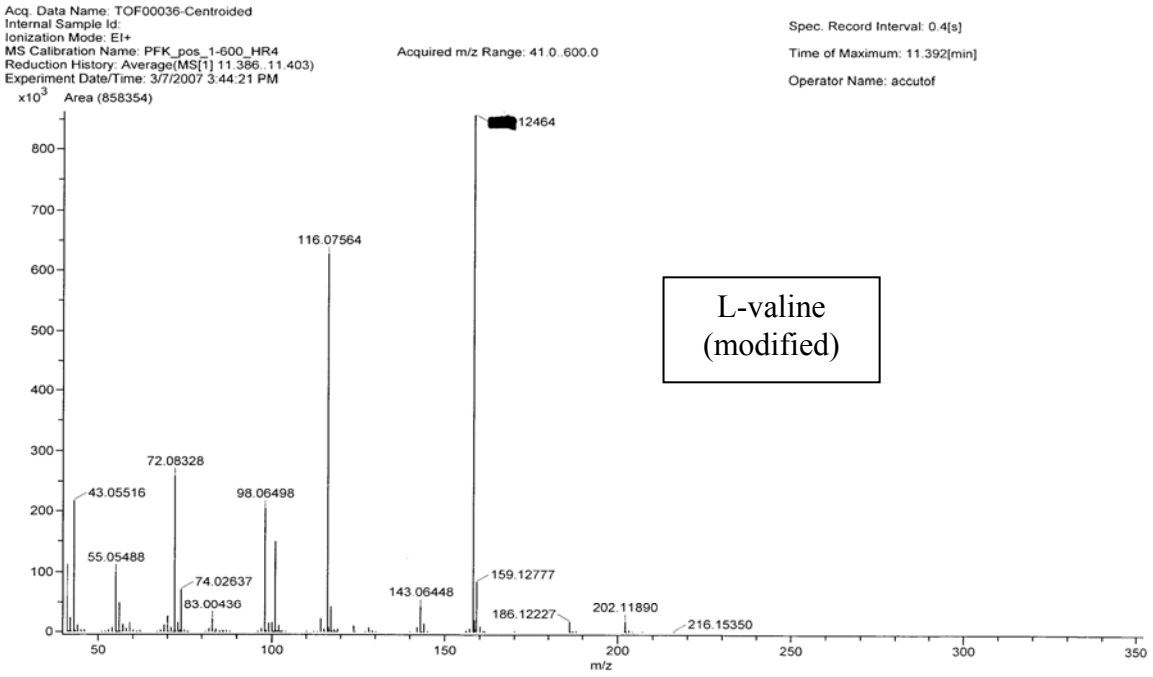

Figure A.7: Diagram of the mass spectrometry fragments (MS; performed at the Organic Chemistry, TU Braunschweig) of **modified L-valine (see Figure 4.15)** obtained through GC-MS from the sample taken after 120 h cultivation time (for the cultivation see Figure 4.10 / chapter 4.4.1)

Curriculum vitae / Lebenslauf

Persönliche Daten

Name:	Hajo Kampe Reershemius
Geburtsdatum / -ort:	30.08.1978 in Norden (Ostfriesland)
Staatsangehörigkeit:	Deutsch
Adresse:	Nordstrasse 29, 38106 Braunschweig
Familienstand:	ledig

Promotion

02.2005 – 06.2008	Wissenschaftlicher Mitarbeiter zum Zwecke der Promotion am Institut für Biochemie und Biotechnologie, TU Braunschweig

Studium

10.1999 – 01.2005	Studium der Biotechnologie (Diplom) Technische Universität Braunschweig
27.09.2001	Vordiplom Biotechnologie
11.01.2005	Diplom Biotechnologie (Dipl.-Biotechnol.)

Schulausbildung

08.1985 – 07.1989	Grundschule Krummhörn – Süd
08.1989 – 06.1991	Orientierungsstufe Emden Wybelsum
08.1991 – 06.1998	Johannes Althusius Gymnasium Emden
22.06.1998	Abitur

Tätigkeiten

08.1998 – 08.1999	Zivildienst beim Deutschen Roten Kreuz Kreisverband Emden